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(71) Applicant (for all designated States except US): SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): ISRAELI, Ron, S. [US/US]; 528 Liberty Avenue, Staten Island, NY 10305 (US). HESTON, Warren, D., W. [US/US]; Apartment 18B, 400 East 85th Street, New York, NY 10028 (US). FAIR, William, R. [US/US]; Apartment 3501, 400 East 70th Street, New York, NY 10021 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham L.L.P., 1185 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

(57) Abstract

This invention provides an isolated mammalian nucleic acid molecule encoding an alternatively spliced prostate-specific membrane (PSM') antigen. This invention provides an isolated nucleic acid molecule encoding a prostate-specific membrane antigen promoter. This invention provides a method of detecting hematogenous micrometastic tumor cells of a subject, and determining prostate cancer progression in a subject.

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

This application is a continuation-in-part of United States Application Serial Nos. 08/466,381 and 08/470,735, both filed June 2, 1995, which are continuations of U.S. Serial No. 08/394,152, filed February 24, 1995, the contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Throughout this application various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each set of Examples in the Experimental Details section.

Prostate cancer is among the most significant medical problems in the United States, as the disease is now the most common malignancy diagnosed in American males. In 1992 there were over 132,000 new cases of prostate cancer detected with over 36,000 deaths attributable to the disease, representing a 17.3% increase over 4 years (2). Five year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. The

rapid increase in the number of cases appears to result
in part from an increase in disease awareness as well
as the widespread use of clinical markers such as the
secreted proteins prostate-specific antigen (PSA) and
5   prostatic acid phosphatase (PAP) (37).

The prostate gland is a site of significant pathology
affected by conditions such as benign growth (BPH),
neoplasia   (prostatic   cancer)   and   infection
10   (prostatitis). Prostate cancer represents the second
leading cause of death from cancer in man (1). However
prostatic cancer is the leading site for cancer
development in men. The difference between these two
facts relates to prostatic cancer occurring with
15   increasing frequency as men age, especially in the ages
beyond 60 at a time when death from other factors often
intervenes.   Also,   the   spectrum   of   biologic
aggressiveness of prostatic cancer is great, so that in
some men following detection the tumor remains a latent
20   histologic tumor and does not become clinically
significant, whereas in other it progresses rapidly,
metastasizes and kills the man in a relatively short 2-
5 year period (1, 3).

25   In prostate cancer cells, two specific proteins that
are made in very high concentrations are prostatic acid
phosphatase (PAP) and prostate specific antigen (PSA)
(4, 5, 6). These proteins have been characterized and
have been used to follow response to therapy. With the
30   development of cancer, the normal architecture of the
gland becomes altered, including loss of the normal
duct structure for the removal of secretions and thus
the secretions reach the serum. Indeed measurement of
serum PSA is suggested as a potential screening method
35   for prostatic cancer. Indeed, the relative amount of
PSA and/or PAP in the cancer reduces as compared to
normal or benign tissue.

PAP was one of the earliest serum markers for detecting metastatic spread (4). PAP hydrolyses tyrosine phosphate and has a broad substrate specificity. Tyrosine phosphorylation is often increased with oncogenic transformation. It has been hypothesized that during neoplastic transformation there is less phosphatase activity available to inactivate proteins that are activated by phosphorylation on tyrosine residues. In some instances, insertion of phosphatases that have tyrosine phosphatase activity has reversed the malignant phenotype.

PSA is a protease and it is not readily appreciated how loss of its activity correlates with cancer development (5, 6). The proteolytic activity of PSA is inhibited by zinc. Zinc concentrations are high in the normal prostate and reduced in prostatic cancer. Possibly the loss of zinc allows for increased proteolytic activity by PSA. As proteases are involved in metastasis and some proteases stimulate mitotic activity, the potentially increased activity of PSA could be hypothesized to play a role in the tumors metastases and spread (7).

Both PSA and PAP are found in prostatic secretions. Both appear to be dependent on the presence of androgens for their production and are substantially reduced following androgen deprivation.

Prostate-specific membrane antigen (PSM) which appears to be localized to the prostatic membrane has been identified. This antigen was identified as the result of generating monoclonal antibodies to a prostatic cancer cell, LNCaP (8).

Dr. Horoszewicz established a cell line designated LNCaP from the lymph node of a hormone refractory,

heavily pretreated patient (9). This line was found to have an aneuploid human male karyotype. It maintained prostatic differentiation functionality in that it produced both PSA and PAP. It possessed an androgen
5   receptor of high affinity and specificity. Mice were immunized with LNCaP cells and hybridomas were derived from sensitized animals. A monoclonal antibody was derived and was designated 7E11-C5 (8). The antibody staining was consistent with a membrane location and
10   isolated fractions of LNCaP cell membranes exhibited a strongly positive reaction with immunoblotting and ELISA techniques. This antibody did not inhibit or enhance the growth of LNCaP cells in vitro or in vivo. The antibody to this antigen was remarkably specific to
15   prostatic epithelial cells, as no reactivity was observed in any other component. Immunohistochemical staining of cancerous epithelial cells was more intense than that of normal or benign epithelial cells.

20   Dr. Horoszewicz also reported detection of immunoreactive material using 7E11-C5 in serum of prostatic cancer patients (8). The immunoreactivity was detectable in nearly 60% of patients with stage D-2 disease and in a slightly lower percentage of patients
25   with earlier stage disease, but the numbers of patients in the latter group are small. Patients with benign prostatic hyperplasia (BPH) were negative. Patients with no apparent disease were negative, but 50-60% of patients in remission yet with active stable disease or
30   with progression demonstrated positive serum reactivity. Patients with non prostatic tumors did not show immunoreactivity with 7E11-C5.

The 7E11-C5 monoclonal antibody is currently in
35   clinical trials. The aldehyde groups of the antibody were oxidized and the linker-chelator glycol-tyrosyl-(n, ε-diethylenetriamine-pentacetic acid)-lysine (GYK-

DTPA) was coupled to the reactive aldehydes of the heavy chain (10). The resulting antibody was designated CYT-356. Immunohistochemical staining patterns were similar except that the CYT-356 modified antibody stained skeletal muscle. The comparison of CYT-356 with 7E11-C5 monoclonal antibody suggested both had binding to type 2 muscle fibers. The reason for the discrepancy with the earlier study, which reported skeletal muscle to be negative, was suggested to be due to differences in tissue fixation techniques. Still, the most intense and definite reaction was observed with prostatic epithelial cells, especially cancerous cells. Reactivity with mouse skeletal muscle was detected with immunohistochemistry but not in imaging studies. The Indium[111]-labeled antibody localized to LNCaP tumors grown in nude mice with an uptake of nearly 30% of the injected dose per gram tumor at four days. In-vivo, no selective retention of the antibody was observed in antigen negative tumors such as PC-3 and DU-145, or by skeletal muscle. Very little was known about the PSM antigen. An effort at purification and characterization has been described at meetings by Dr. George Wright and colleagues (11, 12).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Signal in lane 2 represent the 100kD PSM antigen. The EGFr was used as the positive control and is shown in lane 1. Incubation with rabbit antimouse (RAM) antibody alone served as negative control and is shown in lane 3.

Figures 2A-2D: Upper two photos show LNCaP cytospins staining positively for PSM antigen. Lower left in DU-145 and lower right is PC-3 cytospin, both negative for PSM antigen expression.

Figures 3A-3D: Upper two panels are human prostate sections (BPH) staining positively for PSM antigen. The lower two panels show invasive prostate carcinoma human sections staining positively for expression of the PSM antigen.

Figure 4: 100kD PSM antigen following immunoprecipitation of ^{35}S-Methionine labelled LNCaP cells with Cyt-356 antibody.

Figure 5: 3% agarose gels stained with Ethidium bromide revealing PCR products obtained using the degenerate PSM antigen primers. The arrow points to sample IN-20, which is a 1.1 kb PCR product which was later confirmed to be a partial cDNA coding for the PSM gene.

Figur s 6A-6B: 2% agarose gels of plasmid DNA

resulting from TA cloning of PCR products. Inserts are excised from the PCR II vector (Invitrogen Corp.) by digestion with EcoRI. 1.1 kb PSM gene partial cDNA product is shown in lane 3 of gel 1.

Figure 7: Autoradiogram showing size of cDNA represented in applicants' LNCaP library using M-MLV reverse transcriptase.

Figure 8: Restriction analysis of full-length clones of PSM gene obtained after screening cDNA library. Samples have been cut with Not I and Sal I restriction enzymes to liberate the insert.

Figure 9: Plasmid Southern autoradiogram of full length PSM gene clones. Size is approximately 2.7 kb.

Figure 10: Northern blot revealing PSM expression limited to LNCaP prostate cancer line and H26 Ras-transfected LNCaP cell line. PC-3, DU-145, T-24, SKRC-27, HELA, MCF-7, HL-60, and others were are all <u>negative</u>.

Figure 11: Autoradiogram of Northern analysis revealing expression of 2.8 kb PSM message unique to the LNCaP cell line (lane 1), and absent from the DU-145 (lane 2) and PC-3 cell lines (lane 3). RNA size ladder is shown on the left (kb), and 28S and 18S ribosomal RNA

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bands are indicated on the right.

Figures 12A-12B:

Results of PCR of human prostate tissues using PSM gene primers. Lanes are numbered from left to right. Lane 1, LNCaP; Lane 2, H26; Lane 3, DU-145; Lane 4, Normal Prostate; Lane 5, BPH; Lane 6, Prostate Cancer; Lane 7, BPH; Lane 8, Normal; Lane 9, BPH; Lane 10, BPH; Lane 11, BPH; Lane 12, Normal; Lane 13, Normal; Lane 14, Cancer; Lane 15, Cancer; Lane 16, Cancer; Lane 17, Normal; Lane 18, Cancer; Lane 19, IN-20 Control; Lane 20, PSM cDNA

Figure 13:   Isoelectric point of PSM antigen (non-glycosylated)

Figures 14:1-8 Secondary structure of PSM antigen

Figures 15A-15B:

A. Hydrophilicity plot of PSM antigen
B. Prediction of membrane spanning segments

Figures 16:1-11

Homology with chicken, rat and human transferrin receptor sequence.

Figures 17A-17C:

Immunohistochemical detection of PSM antigen expression in prostate cell lines. Top panel reveals uniformly high level of expression in LNCaP cells; middle panel and lower panel are DU-145 and PC-3 cells respectively,

both negative.

Figure 18:   Autoradiogram of protein gel revealing
              products of PSM coupled *in-vitro*
5   transcription/translation.   Non-
              glycosylated PSM polypeptide is seen at
              84 kDa (lane 1) and PSM glycoprotein
              synthesized following the addition of
              microsomes is seen at 100 kDa (lane 2).

10

Figure 19:   Western Blot analysis detecting PSM
              expression   in   transfected   non-PSM
              expressing PC-3 cells.   100 kDa PSM
              glycoprotein species is clearly seen in
15   LNCaP membranes (lane 1), LNCaP crude
              lysate (lane 2), and PSM-transfected
              PC-3   cells   (lane   4),   but   is
              undetectable in native PC-3 cells (lane
              3).

20

Figure 20:   Autoradiogram   of   ribonuclease
              protection gel assaying for PSM mRNA
              expression in normal human tissues.
              Radiolabeled 1 kb DNA ladder (Gibco-
25   BRL) is shown in lane 1.   Undigested
              probe is 400 nucleotides (lane 2),
              expected protected PSM band is 350
              nucleotides, and tRNA control is shown
              (lane 3).   A strong signal is seen in
30   human prostate (lane 11), with very
              faint, but detectable signals seen in
              human brain (lane 4) and human salivary
              gland (lane 12).

35   Figure 21:   Autoradiogram   of   ribonuclease
              protection gel assaying for PSM mRNA
              expression in LNCaP tumors grown in

nude mice, and in human prostatic tissues. ^{32}P-labeled 1 kb DNA ladder is shown in lane 1. 298 nucleotide undigested probe is shown (lane 2), and tRNA control is shown (lane 3). PSM mRNA expression is clearly detectable in LNCaP cells (lane 4), orthotopically grown LNCaP tumors in nude mice with and without matrigel (lanes 5 and 6), and subcutaneously implanted and grown LNCaP tumors in nude mice (lane 7). PSM mRNA expression is also seen in normal human prostate (lane 8), and in a moderately differentiated human prostatic adenocarcinoma (lane 10). Very faint expression is seen in a sample of human prostate tissue with benign hyperplasia (lane 9).

Figure 22: Ribonuclease protection assay for PSM expression in LNCaP cells treated with physiologic doses of various steroids for 24 hours. ^{32}P-labeled DNA ladder is shown in lane 1. 298 nucleotide undigested probe is shown (lane 2), and tRNA control is shown (lane 3). PSM mRNA expression is highest in untreated LNCaP cells in charcoal-stripped media (lane 4). Applicant see significantly diminished PSM expression in LNCaP cells treated with DHT (lane 5), Testosterone (lane 6), Estradiol (lane 7), and Progesterone (lane 8), with little response to Dexamethasone (lane 9).

Figur 23: Data illustrating results of PSM DNA

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and RNA presence in transfect Dunning
cell lines employing Southern and
Northern blotting techniques

5   **Figures 24A-24B:**

Figure A indicates the power of
cytokine transfected cells to teach
unmodified cells. Administration was
directed to the parental flank or
10   prostate cells. The results indicate
the microenvironment considerations.

Figure B indicates actual potency at a
particular site. The tumor was
15   implanted in prostate cells and treated
with immune cells at two different
sites.

Figures 25A-25B:

20   Relates potency of cytokines in
inhibiting growth of primary tumors.
Animals administered un-modified
parental tumor cells and administered
as a vaccine transfected cells.
25   Following prostatectomy of rodent tumor
results in survival increase.

Figure 26:   PCR amplification with nested primers
improved the level of detection of
30   prostatic cells from approximately one
prostatic cell per 10,000 MCF-7 cells
to better than one cell per million
MCF-7 cells, using either PSA.

35   **Figure 27:**   PCR amplification with nested primers
improved the level of detection of
prostatic cells from approximately one

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prostatic cell per 10,000 MCF-7 cells to better than one cell per million MCF-7 cells, using PSM-derived primers.

5   **Figure 28:**   A representative ethidium stained gel photograph for PSM-PCR. Samples run in lane A represent PCR products generated from the outer primers and samples in lanes labeled B are products of inner

10   primer pairs.

     Figure 29:   PSM Southern blot autoradiograph. The sensitivity of the Southern blot analysis exceeded that of ethidium

15   staining, as can be seen in several samples where the outer product is not visible on figure 3, but is detectable by Southern blotting as shown in figure 4.

20

     Figure 30:   Characteristics of the 16 patients analyzed with respect to their clinical stage, treatment, serum PSA and PAP values, and results of assay.

25

     Figures 31A-31D:
                         The DNA sequence of the 3 kb XhoI fragment of p683 which includes 500 bp of DNA from the RNA start site was

30   determined Sequence 683XFRVS starts from the 5' distal end of PSM promoter.

     Figure 32:   Potential binding sites on the PSM promoter.

35

     Figure 33:   Promoter activity of PSM up-stream fragment/CAT gene chimera.

Figure 34:   Comparison between PSM and PSM' cDNA.
Sequence of the 5' end of PSM cDNA (5)
is shown. Underlined region denotes
nucleotides which are present in PSM
5   cDNA sequence but absent in PSM' cDNA.
Boxed region represents the putative
transmembrane domain of PSM antigen.
*   Asterisk denotes the putative
translation initiation site for PSM'.

10

Figure 35:   Graphical representation of PSM and
PSM' cDNA sequences and antisense PSM
RNA probe (b). PSM cDNA sequence with
complete coding region (5). (a) PSM'
15   cDNA sequence from this study. (c)
Cross hatched and open boxes denote
sequences identity in PSM and PSM'.
Hatched box indicates sequence absent
from PSM'. Regions of cDNA sequence
20   complementary to the antisense probe
are indicated by dashed lines between
the sequences.

Figure 36:   RNase protection assay with PSM
25   specific probe in primary prostatic
tissues.   Total cellular RNA was
isolated from human prostatic samples:
normal prostate, BPH, and CaP. PSM and
PSM' spliced variants are indicated
30   with arrows at right. The left lane is
a DNA ladder. Samples from different
patients are classified as: lanes 3-6,
CaP, carcinoma of prostate; *BPH*, benign
prostatic hypertrophy, lanes 7-9;
35   *normal*, normal prostatic tissue, lanes
10-12. Autoradiograph was exposed for
longer period to read lanes 5 and 9.

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Figure 37:   Tumor Index, a quantification of the
                expression of PSM and PSM'. Expression
                of PSM and PSM' (Fig.3) was quantified
                by densitometry and expressed as a
5   ratio of PSM/PSM' on the Y-axis. Three
                samples each were quantitated for
                primary CaP, BPH and normal prostate
                tissues. Two samples were quantitated
                for LNCaP. *Normal*, normal prostate
10   tissue.

Figure 38:   Characterization of PSM membrane bound
                and PSM' in the cytosol.

15   Figure 39:   Intron 1F: Forward Sequence. Intron 1
                contains a number of trinucleotide
                repeats which can be area associated
                with chromosomal instability in tumor
                cells. LNCaP cells and primary prostate
20   tissue are identical, however in the
                PC-3 and Du-145 tumors they have
                substantially altered levels of these
                trinucleotide repeats which may relate
                to their lack of expression of PSM.

25
     Figures 40A-40B:
                Intron 1R: Reverse Sequence

     Figure 41:   Intron 2F: Forward Sequence
30
     Figure 42:   Intron 2R: Reverse Sequence

     Figures 43A-43B:
                Intron 3F: Forward Sequence
35   Figur s 44A-44B:
                Intron 3R: Reverse Sequence

     Figur s 45A-45B:

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Intron 4F: Forward Sequence

Figures 46A-46B:

Intron 4R: Reverse Sequence

5

Figures 47A-47D:

Sequence of the genomic region upstream
of the 5' transcription start site of
PSM.

10

Figure 48:   Photograph of ethidium bromide stained
gel depicting representative negative
and positive controls used in the
study.   Samples   1-5   were   from,
respectively: male with prostatis, a
15   healthy female volunteer, a male with
BPH, a control 1:1,000,000 dilution of
LNCaP cells, and a patient with renal
cell carcinoma. Below each reaction is
20   the   corresponding   control   reaction
performed   with   beta-2-microglobulin
primers to assure RNA integrity.   No
PCR products were detected for any of
these negative controls.

25

Figure 49:   Photograph   of   gel   displaying
representative   positive   PCR   results
using PSM primers in selected patients
with either localized or disseminated
30   prostate cancer. Sample 1-5 were from.
respectively: a patient with clinically
localized stage $T1_c$ disease, a radical
prostatectomy   patient   with   organ
confined disease and a negative serum
35   PSA, a radical prostatectomy patient
with locally advanced disease and a
negative serum PSA, a patient with

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treated stage D2 disease, and a patient
with treated hormone refractory
disease.

5   **Figure 50:**   Chromosomal location of PSM based on
cosmid construction.

Figure 51:   Human monochromosomal somatic cell
hybrid blot showing that chromosome 11
10   contained the PSM genetic sequence by
Southern analysis. DNA panel digested
with PstI restriction enzyme and probed
with PSM cDNA. Lanes M and H refer to
mouse and hamster DNAs. The numbers
15   correspond to the human chromosomal DNA
in that hybrid.

Figure 52:   Ribonuclease protection assay using PSM
radiolabeled RNA probe revels an
20   abundant PSM mRNA expression in AT6.1-
11 clone 1, but not in AT6.1-11 clone
2, thereby mapping PSM to 11p11.2-13
region.

25   **Figure 53:**   Tissue specific expression of PSM RNA
by Northern blotting and RNAse
protection assay.

Figure 54:   Mapping of the PSM gene to the 11p11.2-
30   p13 region of human chromosome 11 by
southern blotting and in-situ
hybridization.

Figure 55:   Schematic of potential response
35   elements.

Figure 56:   Genomic organization of PSM gene.

Figure 57:   Schematic of metastatic prostate cell

Figure 58A-58C:

Nucleic acid of PSM genomic DNA is read
5 prime away from the transcription
start site: number on the sequences
indicates nucleotide upstream from the
start site. Therefore, nucleotide #121
is actually -121 using conventional
numbering system.

Figure 59:

Representation of NAAG 1, acividin,
azotomycin, and 6-diazo-5-oxo-
norleucine, DON.

Figure 60:

Preparation of N-
acetylaspartylglutamate, NAAG 1.

Figure 61:

Synthesis of N-acetylaspartylglutamate,
NAAG 1.

Figure 62:

Synthesis of N-phosphonoacetylaspartyl-
L-glutamate.

Figure 63:

Synthesis of 5-diethylphosphonon-2
amino benzylvalerate intermediate.

Figure 64:

Synthesis of analog 4 and 5.

Figure 65:

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Representation of DON, analogs 17-20.

Figures 77A-77B:

           Intron 3R: Reverse Sequence

5   **Figures 78A-78C:**

           Intron 4F: Forward Sequence

Figures 79A-79E:

           Intron 4RF: Reverse Sequence

10

Figure 80:

           PSM genomic organization of the exons
and 19 intron junction sequences. The
exon/intron junctions (See Example 15)

15   are as follows:

           1.   Exon /intron 1 at bp 389-390;

           2.   Exon /intron 2 at bp 490-491;

           3.   Exon /intron 3 at bp 681-682;

           4.   Exon /intron 4 at bp 784-785;

20   5.   Exon /intron 5 at bp 911-912;

           6.   Exon /intron 6 at bp 1096-1097;

           7.   Exon /intron 7 at bp 1190-1191;

           8.   Exon /intron 8 at bp 1289- 1290;

           9.   Exon /intron 9 at bp 1375-1376;

25   10.   Exon /intron 10 at bp 1496-1497;

           11.   Exon /intron 11 at bp 1579-1580;

           12.   Exon /intron 12 at bp 1640-1641;

           13.   Exon /intron 13 at bp 1708-1709;

           14.   Exon /intron 14 at bp 1803-1804;

30   15.   Exon /intron 15 at bp 1892-1893;

           16.   Exon /intron 16 at bp 2158-2159;

           17.   Exon /intron 17 at bp 2240-2241;

           18.   Exon /intron 18 at bp 2334-2335;

           19.   Exon /intron 19 at bp 2644-2645.

35

SUMMARY OF THE INVENTION

This invention provides an isolated mammalian nucleic
acid molecule encoding an alternatively spliced
prostate-specific membrane (PSM') antigen.

This invention provides an isolated nucleic acid
molecule encoding a prostate-specific membrane antigen
promoter.   This invention provides a method of
detecting hematogenous micrometastic tumor cells of a
subject, and determining prostate cancer progression
in a subject.

Detailed Description of the Invention

Throughout this application, references to specific
nucleotides are to nucleotides present on the coding
5   strand of the nucleic acid. The following standard
abbreviations are used throughout the specification to
indicate specific nucleotides:

C=cytosine   A=adenosine
10   T=thymidine   G=guanosine

A "gene" means a nucleic acid molecule, the sequence of
which includes all the information required for the
normal regulated production of a particular protein,
15   including the structural coding sequence, promoters and
enhancers.

This invention provides an isolated mammalian nucleic
acid encoding an alternatively spliced prostate-
20   specific membrane (PSM') antigen.

This invention provides an isolated mammalian nucleic
acid encoding a mammalian prostate-specific membrane
(PSM) antigen.
25
This invention further provides an isolated mammalian
DNA molecule of an isolated mammalian nucleic acid
molecule encoding an alternatively spliced prostate-
specific membrane antigen.   This invention also
30   provides an isolated mammalian cDNA molecule encoding
a mammalian alternatively spliced prostate-specific
membrane antigen. This invention provides an isolated
mammalian RNA molecule encoding a mammalian
alternatively spliced prostate-specific cytosolic
35   antigen.

This invention further provides an isolated mammalian

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DNA molecule of an isolated mammalian nucleic acid
molecule encoding a mammalian prostate-specific
membrane antigen. This invention also provides an
isolated mammalian cDNA molecule encoding a mammalian
5   prostate-specific membrane antigen. This invention
provides an isolated mammalian RNA molecule encoding a
mammalian prostate-specific membrane antigen.

In the preferred embodiment of this invention, the
10   isolated nucleic sequence is cDNA from human as shown
in Figures 47A-47D. This human sequence was submitted
to GenBank (Los Alamos National Laboratory, Los Alamos,
New Mexico) with Accession Number, M99487 and the
description as PSM, Homo sapiens, 2653 base-pairs.
15

This invention also encompasses DNAs and cDNAs which
encode amino acid sequences which differ from those of
PSM or PSM' antigen, but which should not produce
phenotypic changes. Alternatively, this invention also
20   encompasses DNAs and cDNAs which hybridize to the DNA
and cDNA of the subject invention. Hybridization
methods are well known to those of skill in the art.

For example, high stringent hybridization conditions
25   are selected at about 5° C lower than the thermal
melting point (Tm) for the specific sequence at a
defined ionic strength and pH. The Tm is the
temperature (under defined ionic strength and pH) at
which 50% of the target sequence hybridizes to a
30   perfectly matched probe. Typically, stringent
conditions will be those in which the salt
concentration is at least about 0.02 molar at pH 7 and
the temperature is at least about 60°C. As other
factors may significantly affect the stringency of
35   hybridization, including, among others, base
composition and size of the complementary strands, the
presence of organic solvents, ie. salt or formamide

concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. For Example high stringency may be attained for example by overnight
5   hybridization at about 68°C in a 6x SSC solution, washing at room temperature with 6x SSC solution, followed by washing at about 68°C in a 6x SSC in a 0.6x SSX solution.

10   Hybridization with moderate stringency may be attained for example by: 1) filter pre-hybridizing and hybridizing with a solution of 3x sodium chloride, sodium citrate (SSC), 50% formamide, 0.1M Tris buffer at Ph 7.5, 5x Denhardt's solution;   2.) pre-
15   hybridization at 37°C for 4 hours; 3) hybridization at 37°C with amount of labelled probe equal to 3,000,000 cpm total for 16 hours; 4) wash in 2x SSC and 0.1% SDS solution; 5) wash 4x for 1 minute each at room temperature at 4x at 60°C for 30 minutes each; and 6)
20   dry and expose to film.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide
25   and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and
30   useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated mammalian nucleic acid molecules
35   encoding a mammalian prostate-specific membrane antigen and the alternatively spliced PSM' are useful for the development of probes to study the tumorigenesis of

prostate cancer.

This invention also provides an isolated nucleic acid
molecule of at least 15 nucleotides capable of
5   specifically hybridizing with a sequence of a nucleic
acid molecule encoding the prostate-specific membrane
antigen or the alternatively spliced prostate specific
membrane antigen.

10   This nucleic acid molecule produced can either be DNA
or RNA. As used herein, the phrase "specifically
hybridizing" means the ability of a nucleic acid
molecule to recognize a nucleic acid sequence
complementary to its own and to form double-helical
15   segments through hydrogen bonding between complementary
base pairs.

This nucleic acid molecule of at least 15 nucleotides
capable of specifically hybridizing with a sequence of
20   a nucleic acid molecule encoding the prostate-specific
membrane antigen can be used as a probe. Nucleic acid
probe technology is well known to those skilled in the
art who will readily appreciate that such probes may
vary greatly in length and may be labeled with a
25   detectable label, such as a radioisotope or fluorescent
dye, to facilitate detection of the probe. DNA probe
molecules may be produced by insertion of a DNA
molecule which encodes PSM antigen into suitable
vectors, such as plasmids or bacteriophages, followed
30   by transforming into suitable bacterial host cells,
replication in the transformed bacterial host cells and
harvesting of the DNA probes, using methods well known
in the art. Alternatively, probes may be generated
chemically from DNA synthesizers.
35

RNA probes may be generated by inserting the PSM
antigen molecule downstream of a bacteriophage promoter

such as T3, T7 or SP6. Large amounts of RNA probe may
be produced by incubating the labeled nucleotides with
the linearized PSM antigen fragment where it contains
an upstream promoter in the presence of the appropriate
5   RNA polymerase.

This invention also provides a nucleic acid molecule of
at least 15 nucleotides capable of specifically
hybridizing with a sequence of a nucleic acid molecule
10   which is complementary to the mammalian nucleic acid
molecule encoding a mammalian prostate-specific
membrane antigen. This molecule may either be a DNA or
RNA molecule.

15   The current invention further provides a method of
detecting the expression of a mammalian PSM or PSM'
antigen expression in a cell which comprises obtaining
total mRNA from the cell, contacting the mRNA so
obtained with a labelled nucleic acid molecule of at
20   least 15 nucleotides capable of specifically
hybridizing with a sequence of the nucleic acid
molecule encoding a mammalian PSM or PSM' antigen under
hybridizing conditions, determining the presence of
mRNA hybridized to the molecule and thereby detecting
25   the expression of the mammalian prostate-specific
membrane antigen in the cell. The nucleic acid
molecules synthesized above may be used to detect
expression of a PSM or PSM' antigen by detecting the
presence of mRNA coding for the PSM antigen. Total
30   mRNA from the cell may be isolated by many procedures
well known to a person of ordinary skill in the art.
The hybridizing conditions of the labelled nucleic acid
molecules may be determined by routine experimentation
well known in the art. The presence of mRNA hybridized
35   to the probe may be determined by gel electrophoresis
or other methods known in the art. By measuring the
amount of the hybrid made, the expression of the PSM

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antigen by the cell can be determined.   The labeling
may be radioactive.   For an example, one or more
radioactive nucleotides can be incorporated in the
nucleic acid when it is made.

5

In one embodiment of this invention, nucleic acids are
extracted by precipitation from lysed cells and the
mRNA is isolated from the extract using an oligo-dT
column which binds the poly-A tails of the mRNA

10   molecules (13).   The mRNA is then exposed to
radioactively labelled probe on a nitrocellulose
membrane, and the probe hybridizes to and thereby
labels complementary mRNA sequences.   Binding may be
detected by luminescence autoradiography or

15   scintillation counting.   However, other methods for
performing these steps are well known to those skilled
in the art, and the discussion above is merely an
example.

20   This invention further provides another method to
detect expression of a PSM or PSM' antigen in tissue
sections which comprises contacting the tissue sections
with a labelled nucleic acid molecule of at least 15
nucleotides capable of specifically hybridizing with a

25   sequence of nucleic acid molecules encoding a mammalian
PSM antigen under hybridizing conditions, determining
the presence of mRNA hybridized to the molecule and
thereby detecting the expression of the mammalian PSM
or PSM' antigen in tissue sections.   The probes are

30   also useful for in-situ hybridization or in order to
locate tissues which express this gene, or for other
hybridization assays for the presence of this gene or
its mRNA in various biological tissues.   The in-situ
hybridization using a labelled nucleic acid molecule is

35   well known in the art.   Essentially, tissue sections
are incubated with the labelled nucleic acid molecule
to allow the hybridization to occur.   The molecule will

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carry a marker for the detection because it is "labelled", the amount of the hybrid will be determined based on the detection of the amount of the marker and so will the expression of PSM antigen.

This invention further provides isolated PSM or PSM' antigen nucleic acid molecule operatively linked to a promoter of RNA transcription. The isolated PSM or PSM' antigen sequence can be linked to vector systems. Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners. This invention further provides a vector which comprises the isolated nucleic acid molecule encoding for the PSM or PSM' antigen.

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

In an embodiment, the PSM sequence is cloned in the Not I/Sal I site of pSPORT/vector (Gibco® - BRL). This plasmid, p55A-PSM, was deposited on August 14, 1992 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, p55A-PSM, was accorded ATCC Accession Number 75294.

This invention further provides a host vector system
for the production of a polypeptide having the
biological activity of the prostate-specific membrane
antigen. These vectors may be transformed into a
5   suitable host cell to form a host cell vector system
for the production of a polypeptide having the
biological activity of PSM antigen.

Regulatory elements required for expression include
10   promoter sequences to bind RNA polymerase and
transcription initiation sequences for ribosome
binding. For example, a bacterial expression vector
includes a promoter such as the lac promoter and for
transcription initiation the Shine-Dalgarno sequence
15   and the start codon AUG (14). Similarly, a eukaryotic
expression vector includes a heterologous or homologous
promoter for RNA polymerase II, a downstream
polyadenylation signal, the start codon AUG, and a
termination codon for detachment of the ribosome. Such
20   vectors may be obtained commercially or assembled from
the sequences described by methods well known in the
art, for example the methods described above for
constructing vectors in general. Expression vectors
are useful to produce cells that express the PSM
25   antigen.

This invention further provides an isolated DNA or cDNA
molecule described hereinabove wherein the host cell is
selected from the group consisting of bacterial cells
30   (such as E.coli), yeast cells, fungal cells, insect
cells and animal cells. Suitable animal cells include,
but are not limited to Vero cells, HeLa cells, Cos
cells, CV1 cells and various primary mammalian cells.

35   This invention further provides a method of producing
a polypeptide having the biological activity of the
prostate-specific membrane antigen which comprising

growing host cells of a vector system containing the
PSM antigen sequence under suitable conditions
permitting production of the polypeptide and recovering
the polypeptide so produced.

5

This invention provides a mammalian cell comprising a
DNA molecule encoding a mammalian PSM or PSM' antigen,
such as a mammalian cell comprising a plasmid adapted
for expression in a mammalian cell, which comprises a
10   DNA molecule encoding a mammalian PSM antigen and the
regulatory elements necessary for expression of the DNA
in the mammalian cell so located relative to the DNA
encoding the mammalian PSM or PSM' antigen as to permit
expression thereof.

15

Numerous mammalian cells may be used as hosts,
including, but not limited to, the mouse fibroblast
cell NIH3T3, CHO cells, HeLa cells, Ltk⁻ cells, Cos
cells, etc. Expression plasmids such as that described
20   _supra_ may be used to transfect mammalian cells by
methods well known in the art such as calcium phosphate
precipitation, electroporation or DNA encoding the
mammalian PSM antigen may be otherwise introduced into
mammalian cells, e.g., by microinjection, to obtain
25   mammalian cells which comprise DNA, e.g., cDNA or a
plasmid, encoding a mammalian PSM antigen.

This invention provides a method for determining
whether a ligand can bind to a mammalian prostate-
30   specific membrane antigen which comprises contacting a
mammalian cell comprising an isolated DNA molecule
encoding a mammalian prostate-specific membrane antigen
with the ligand under conditions permitting binding of
ligands to the mammalian prostate-specific membrane
35   antigen, and thereby determining whether the ligand
binds to a mammalian prostate-specific membrane
antigen.

This invention further provides ligands bound to the mammalian PSM or PSM' antigen.

5   This invention also provides a therapeutic agent comprising a ligand identified by the above-described method and a cytotoxic agent conjugated thereto. The cytotoxic agent may either be a radioisotope or a toxin. Examples of radioisotopes or toxins are well known to one of ordinary skill in the art.

10

     This invention also provides a method of imaging prostate cancer in human patients which comprises administering to the patients at least one ligand identified by the above-described method, capable of
15   binding to the cell surface of the prostate cancer cell and labelled with an imaging agent under conditions permitting formation of a complex between the ligand and the cell surface PSM or PSM' antigen. This invention further provides a composition comprising an
20   effective imaging agent of the PSM OR PSM' antigen ligand and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to one of ordinary skill in the art. For an example, such a pharmaceutically acceptable carrier can be
25   physiological saline.

     Also provided by this invention is a purified mammalian PSM and PSM' antigen. As used herein, the term "purified prostate-specific membrane antigen" shall
30   mean isolated naturally-occurring prostate-specific membrane antigen or protein (purified from nature or manufactured such that the primary, secondary and tertiary conformation, and posttranslational modifications are identical to naturally-occurring
35   material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e. continuous sequence of amino acid residues).

Such polypeptides include derivatives and analogs.

This invention provides an isolated nucleic acid
molecule encoding a prostate-specific membrane antigen
5   promoter. In one embodiment the PSM promoter has at
least the sequence as in Figures 58A-58C.

This invention provides an isolated nucleic acid
molecule encoding an alternatively spliced prostate-
10   specific membrane antigen promoter.

This invention further provides a polypeptide encoded
by the isolated mammalian nucleic acid sequence of PSM
and PSM' antigen.

15

It is believed that there may be natural ligand
interacting with the PSM or PSM' antigen. This
invention provides a method to identify such natural
ligand or other ligand which can bind to the PSM or
20   PSM' antigen. A method to identify the ligand
comprises a) coupling the purified mammalian PSM or
PSM' antigen to a solid matrix, b) incubating the
coupled purified mammalian PSM or PSM' protein with the
potential ligands under the conditions permitting
25   binding of ligands and the purified PSM or PSM'
antigen; c) washing the ligand and coupled purified
mammalian PSM or PSM' antigen complex formed in b) to
eliminate the nonspecific binding and impurities and
finally d) eluting the ligand from the bound purified
30   mammalian PSM or PSM' antigen. The techniques of
coupling proteins to a solid matrix are well known in
the art. Potential ligands may either be deduced from
the structure of mammalian PSM or PSM' by other
empirical experiments known by ordinary skilled
35   practitioners. The conditions for binding may also
easily be determined and protocols for carrying such
experimentation have long been well documented (15).

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The ligand-PSM antigen complex will be washed. Finally, the bound ligand will be eluted and characterized. Standard ligands characterization techniques are well known in the art.

The above method may also be used to purify ligands from any biological source. For purification of natural ligands in the cell, cell lysates, serum or other biological samples will be used to incubate with the mammalian PSM or PSM' antigen bound on a matrix. Specific natural ligand will then be identified and purified as above described.

With the protein sequence information, antigenic areas may be identified and antibodies directed against these areas may be generated and targeted to the prostate cancer for imaging the cancer or therapies.

This invention provides an antibody directed against the amino acid sequence of a mammalian PSM or PSM' antigen.

This invention provides a method to select specific regions on the PSM or PSM' antigen to generate antibodies. The protein sequence may be determined from the PSM or PSM' DNA sequence. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid

sequences may be selected and used to generate antibodies specific to mammalian PSM antigen. For an example, hydrophilic sequences of the human PSM antigen shown in hydrophilicity plot of Figures 16:1-11 may be

5   easily selected. The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen.

10

Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B

15   cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by _in vitro_ techniques known to a person of ordinary skill in the art. These

20   antibodies are useful to detect the expression of mammalian PSM antigen in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

25   In one embodiment, peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.) of human PSM antigen are selected.

30   This invention further provides polyclonal and monoclonal antibody(ies) against peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.).

35

This invention provides a therapeutic agent comprising antibodies or ligand(s) directed against PSM antigen

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and a cytotoxic agent conjugated thereto or antibodies linked enzymes which activate prodrug to kill the tumor.   The cytotoxic agent may either be a radioisotope or toxin.

5

This invention provides a method of imaging prostate cancer in human patients which comprises administering to the patient the monoclonal antibody directed against the peptide of the mammalian PSM or PSM' antigen

10   capable of binding to the cell surface of the prostate cancer cell and labeled with an imaging agent under conditions permitting formation of a complex between the monoclonal antibody and the cell surface prostate-specific membrane antigen.   The imaging agent is a

15   radioisotope such as Indium^{111}.

This invention further provides a prostate cancer specific imaging agent comprising the antibody directed against PSM or PSM' antigen and a radioisotope

20   conjugated thereto.

This invention also provides a composition comprising an effective imaging amount of the antibody directed against the PSM or PSM' antigen and a pharmaceutically

25   acceptable carrier.   The methods to determine effective imaging amounts are well known to a skilled practitioner.   One method is by titration using different amounts of the antibody.

3C   This invention further provides an immunoassay for measuring the amount of the prostate-specific membrane antigen in a biological sample comprising steps of a) contacting the biological sample with at least one antibody directed against the PSM or PSM' antigen to

35   form a complex with said antibody and the prostate-specific membrane antigen, and b) measuring the amount of the prostate-specific membrane antigen in said

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biological sample by measuring the amount of said complex. One example of the biological sample is a serum sample.

5   This invention provides a method to purify mammalian prostate-specific membrane antigen comprising steps of a) coupling the antibody directed against the PSM or PSM' antigen to a solid matrix; b) incubating the coupled antibody of a) with lysate containing prostate-
10   specific membrane antigen under the condition which the antibody and prostate membrane specific can bind; c) washing the solid matrix to eliminate impurities and d) eluting the prostate-specific membrane antigen from the coupled antibody.

15

        This invention also provides a transgenic nonhuman mammal which comprises the isolated nucleic acid molecule encoding a mammalian PSM or PSM' antigen. This invention further provides a transgenic nonhuman
20   mammal whose genome comprises antisense DNA complementary to DNA encoding a mammalian prostate-specific membrane antigen so placed as to be transcribed into antisense mRNA complementary to mRNA encoding the prostate-specific membrane antigen and
25   which hybridizes to mRNA encoding the prostate specific antigen thereby reducing its translation.

        Animal model systems which elucidate the physiological and behavioral roles of mammalian PSM or PSM' antigen
30   are produced by creating transgenic animals in which the expression of the PSM or PSM' antigen is either increased or decreased, or the amino acid sequence of the expressed PSM antigen is altered, by a variety of techniques. Examples of these techniques include, but
35   are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a mammalian PSM or PSM' antigen, by microinjection, electroporation, retroviral

transfection or other means well known to those skilled
in the art, into appropriate fertilized embryos in
order to produce a transgenic animal (16) or 2)
Homologous recombination (17) of mutant or normal,
5   human or animal versions of these genes with the native
gene locus in transgenic animals to alter the
regulation of expression or the structure of these PSM
or PSM' antigen sequences. The technique of homologous
recombination is well known in the art. It replaces
10   the native gene with the inserted gene and so is useful
for producing an animal that cannot express native PSM
antigen but does express, for example, an inserted
mutant PSM antigen, which has replaced the native PSM
antigen in the animal's genome by recombination,
15   resulting in undere xpression of the transporter.
Microinjection adds genes to the genome, but does not
remove them, and so is useful for producing an animal
which expresses its own and added PSM antigens,
resulting in over expression of the PSM antigens.

20

One means available for producing a transgenic animal,
with a mouse as an example, is as follows: Female
mice are mated, and the resulting fertilized eggs are
dissected out of their oviducts. The eggs are stored
25   in an appropriate medium such as Me medium (16). DNA
or cDNA encoding a mammalian PSM antigen is purified
from a vector by methods well known in the art.
Inducible promoters may be fused with the coding region
of the DNA to provide an experimental means to regulate
30   expression of the trans-gene. Alternatively or in
addition, tissue specific regulatory elements may be
fused with the coding region to permit tissue-specific
expression of the trans-gene. The DNA, in an
appropriately buffered solution, is put into a
35   microinjection needle (which may be made from capillary
tubing using a pipet puller) and the egg to be injected
is put in a depression slide. The needle is inserted

into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Another use of the PSM antigen sequence is to isolate homologous gene or genes in different mammals. The gene or genes can be isolated by low stringency screening of either cDNA or genomic libraries of different mammals using probes from PSM sequence. The positive clones identified will be further analyzed by DNA sequencing techniques which are well known to an ordinary person skilled in the art. For example, the detection of members of the protein serine kinase family by homology probing.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells comprising introducing a DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell of a subject, in a way that expression of the prostate specific membrane antigen is under the control of the regulatory element, thereby suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells. The subject may be a mammal or more specifically a human.

In one embodiment, the DNA molecule encoding prostate specific membrane antigen operatively linked to a 5'

regulatory element forms part of a transfer vector
which is inserted into a cell or organism. In addition
the vector is capable or replication and expression of
prostate specific membrane antigen. The DNA molecule
5   encoding prostate specific membrane antigen can be
integrated into a genome of a eukaryotic or prokaryotic
cell or in a host cell containing and/or expressing a
prostate specific membrane antigen.

10   Further, the DNA molecule encoding prostate specific
membrane antigen may be introduced by a bacterial,
viral, fungal, animal, or liposomal delivery vehicle.
Other means are also available and known to an ordinary
skilled practitioner.
15

Further, the DNA molecule encoding a prostate specific
membrane antigen operatively linked to a promoter or
enhancer. A number of viral vectors have been
described including those made from various promoters
20   and other regulatory elements derived from virus
sources. Promoters consist of short arrays of nucleic
acid sequences that interact specifically with cellular
proteins involved in transcription. The combination of
different recognition sequences and the cellular
25   concentration of the cognate transcription factors
determines the efficiency with which a gene is
transcribed in a particular cell type.

Examples of suitable promoters include a viral
30   promoter. Viral promoters include: adenovirus
promoter, an simian virus 40 (SV40) promoter, a
cytomegalovirus (CMV) promoter, a mouse mammary tumor
virus (MMTV) promoter, a Malony murine leukemia virus
promoter, a murine sarcoma virus promoter, and a Rous
35   sarcoma virus promoter.

Further, another suitable promoter is a heat shock

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promoter. Additionally, a suitable promoter is a bacteriophage promoter. Examples of suitable bacteriophage promoters include but not limited to, a T7 promoter, a T3 promoter, an SP6 promoter, a lambda promoter, a baculovirus promoter.

Also suitable as a promoter is an animal cell promoter such as an interferon promoter, a metallothionein promoter, an immunoglobulin promoter. A fungal promoter is also a suitable promoter. Examples of fungal promoters include but are not limited to, an ADC1 promoter, an ARG promoter, an ADH promoter, a CYC1 promoter, a CUP promoter, an ENO1 promoter, a GAL promoter, a PHO promoter, a PGK promoter, a GAPDH promoter, a mating type factor promoter. Further, plant cell promoters and insect cell promoters are also suitable for the methods described herein.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells, comprising introducing a DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5' regulatory element coupled with a therapeutic DNA into a tumor cell of a subject, thereby suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells. The subject may be a mammal or more specifically a human.

Further, the therapeutic DNA which is coupled to the DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell may code for a cytokine, viral antigen, or a pro-drug activating enzyme. Other means are also available and known to an ordinary skilled

practitioner.

In addition, this invention provides a prostate tumor
cell, comprising a DNA molecule isolated from mammalian
5   nucleic acid encoding a mammalian prostate-specific
membrane antigen under the control of a prostate
specific membrane antigen operatively linked to a 5'
regulatory element.

10   As used herein, DNA molecules include complementary DNA
(cDNA), synthetic DNA, and genomic DNA.

This invention provides a therapeutic vaccine for
preventing human prostate tumor growth or stimulation
15   of prostate tumor cells in a subject, comprising
administering an effective amount to the prostate cell,
and a pharmaceutical acceptable carrier, thereby
preventing the tumor growth or stimulation of tumor
cells in the subject. Other means are also available
20   and known to an ordinary skilled practitioner.

This invention provides a method of detecting
hematogenous micrometastic tumor cells of a subject,
comprising (A) performing nested polymerase chain
25   reaction (PCR) on blood, bone marrow or lymph node
samples of the subject using the prostate specific
membrane antigen primers or alternatively spliced
prostate specific antigen primers, and (B) verifying
micrometastases by DNA sequencing and Southern
30   analysis, thereby detecting hematogenous micrometastic
tumor cells of the subject. The subject may be a mammal
or more specifically a human.

The micrometastatic tumor cell may be a prostatic
35   cancer and the DNA primers may be derived from prostate
specific antigen. Further, the subject may be
administered with simultaneously an effective amount of

hormones, so as to increase expression of prostate specific membrane antigen. Further, growth factors or cytokine may be administered in separately or in conjunction with hormones. Cytokines include, but are not limited to: transforming growth factor beta, epidermal growth factor (EGF) family, fibroblast growth factors, hepatocyte growth factor, insulin-like growth factors, B-nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, interleukin 1, IL-1 receptor antagonist, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, IL-6 soluble receptor, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, interleukin 13, angiogenin, chemokines, colony stimulating factors, granulocyte-macrophage colony stimulating factors, erythropoietin, interferon, interferon gamma, leukemia inhibitory factor, oncostatin M, pleiotrophin, secretory leukocyte protease inhibitor, stem cell factor, tumor necrosis factors, adhesion molecule, and soluble tumor necrosis factor (TNF) receptors.

This invention provides a method of abrogating the mitogenic response due to transferrin, comprising introducing a DNA molecule encoding prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell, the expression of which gene is directly associated with a defined pathological effect within a multicellular organism, thereby abrogating mitogen response due to transferrin. The tumor cell may be a prostate cell.

This invention provides a method of determining prostate cancer progression in a subject which comprises: a) obtaining a suitable prostate tissue sample; b) extracting RNA from the prostate tissue sample; c) performing a RNAse protection assay on the

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RNA thereby forming a duplex RNA-RNA hybrid; d)
detecting PSM and PSM' amounts in the tissue sample; e)
calculating a PSM/PSM' tumor index, thereby determining
prostate cancer progression in the subject. In-situ
5   hyribridization may be performed in conjunction with
the above detection method.

This invention provides a method of detecting prostate
cancer in a subject which comprises: (a) obtaining
10   from a subject a prostate tissue sample;   (b) treating
the tissue sample so as to separately recover nucleic
acid molecules present in the prostate tissue sample;
(c) contacting the resulting nucleic acid molecules
with   multiple   pairs   of   single-stranded   labeled
15   oligonucleotide primers, each such pair being capable
of specifically hybridizing to the tissue sample, under
hybridizing conditions;   (d) amplifying any nucleic
acid molecules to which a pair of primers hybridizes so
as to obtain a double-stranded amplification product;
20   (e) treating any such double-stranded amplification
product so as to obtain single-stranded nucleic acid
molecules therefrom;   (f) contacting any resulting
single-stranded nucleic acid molecules with multiple
single-stranded labeled oligonucleotide probes, each
25   such probe containing the same label and being capable
of specifically hybridizing with such tissue sample,
under hybridizing conditions;   (g) contacting any
resulting hybrids with an antibody to which a marker is
attached and which is capable of specifically forming
30   a complex with the labeled-probe, when the probe is
present in such a complex, under complexing conditions;
and (h) detecting the presence of any resulting
complexes, the presence thereof being indicative of
prostate cancer in a subject.
35

This invention provides a method of enhancing antibody
based targeting of PSM or PSM' in prostate tissue for

diagnosis or therapy of prostate cancer comprising administering to a patient b-FGF in sufficient amount to cause upregulation of PSM or PSM' expression.

5   This invention provides a method of enhancing antibody based targeting of PSM or PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient TGF in sufficient amount to cause upregulation of PSM expression or PSM'.

10

This invention provides a method of enhancing antibody based targeting of PSM or PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient EGF in sufficient amount to

15   cause upregulation of PSM or PSM' expression.

This invention provides a pharmaceutical composition comprising an effective amount of PSM or the alternatively spliced PSM and a carrier or diluent.

20   Further, this invention provides a method for administering to a subject, preferably a human, the pharmaceutical composition. Further, this invention provides a composition comprising an amount of PSM or the alternatively spliced PSM and a carrier or diluent.

25   Specifically, this invention may be used as a food additive.

The compositions are administered in a manner compatible with the dosage formulation, and in a

30   therapeutically effective amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each subject.

35   Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or

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more hour intervals by a subsequent injection or other
administration.

5   As used herein administration means a method of
        administering to a subject. Such methods are well
        known to those skilled in the art and include, but are
        not limited to, administration topically, parenterally,
        orally, intravenously, intramuscularly, subcutaneously
        or by aerosol. Administration of PSM may be effected
10   continuously or intermittently.

        The pharmaceutical formulations or compositions of this
        invention may be in the dosage form of solid, semi-
        solid, or liquid such as, e.g., suspensions, aerosols
15   or the like. Preferably the compositions are
        administered in unit dosage forms suitable for single
        administration of precise dosage amounts. The
        compositions may also include, depending on the
        formulation desired, pharmaceutically-acceptable, non-
20   toxic carriers or diluents, which are defined as
        vehicles commonly used to formulate pharmaceutical
        compositions for animal or human administration. The
        diluent is selected so as not to affect the biological
        activity of the combination. Examples of such diluents
25   are distilled water, physiological saline, Ringer's
        solution, dextrose solution, and Hank's solution. In
        addition, the pharmaceutical composition or formulation
        may also include other carriers, adjuvants; or
        nontoxic, nontherapeutic, nonimmunogenic stabilizers
30   and the like. Effective amounts of such diluent or
        carrier are those amounts which are effective to obtain
        a pharmaceutically acceptable formulation in terms of
        solubility of components, or biological activity, etc

35   This invention will be better understood from the
        Experimental Details which follow. However, one
        skilled in the art will readily appreciate that the

specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

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EXPERIMENTAL DETAILS

EXAMPLE 1:

5   **Materials and Methods:** The approach for cloning the
gene involved purification of the antigen by
immunoprecipitation, and microsequencing of several
internal peptides for use in synthesizing degenerate
oligonucleotide primers for subsequent use in the
10   polymerase chain reaction (19, 20). A partial cDNA was
amplified as a PCR product and this was used as a
homologous probe to clone the full-length cDNA molecule
from a LNCaP (Lymph Node Carcinoma of Prostate) cell
line cDNA plasmid library (8).

15

Western Analysis of the PSM Antigen: Membrane proteins
were isolated from cells by hypotonic lysis followed by
centrifugation over a sucrose density gradient (21).
10-20μg of LNCaP, DU-145, and PC-3 membrane proteins
20   were electrophoresed through a 10% SDS-PAGE resolving
gel with a 4% stacking gel at 9-10 milliamps for 16-18
hours.   Proteins were electroblotted onto PVDF
membranes (Millipore® Corp.) in transfer buffer (48mM
Tris base, 39mM Glycine, 20% Methanol) at 25 volts
25   overnight at 4°C.   Membranes were blocked in TSB (0.15M
NaCl, 0.01M Tris base, 5% BSA) for 30 minutes at room
temperature followed by incubation with 10-15μg/ml of
CYT-356 monoclonal antibody (Cytogen Corp.) for 2
hours.   Membranes were then incubated with 10-15μg/ml
30   of rabbit anti-mouse immunoglobulin (Accurate
Scientific) for 1 hour at room temperature followed by
incubation with ^{125}I-Protein A (Amersham®) at 1x10^6
cpm/ml at room temperature.   Membranes were then washed
and autoradiographed for 12-24 hours at -70°C (Figure
35   1).

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Immunohistochemical Analysis of PSM Antigen Expression:
The avidin-biotin method of immunohistochemical detection was employed to analyze both human tissue sections and cell lines for PSM Antigen expression
5   (22). Cryostat-cut prostate tissue sections (4-6μm thick) were fixed in methanol/acetone for 10 minutes. Cell cytospins were made on glass slides using 50,000 cells/100μl/slide. Samples were treated with 1% hydrogen peroxide in PBS for 10-15 minutes in order to
10   remove any endogenous peroxidase activity. Tissue sections were washed several times in PBS, and then incubated with the appropriate suppressor serum for 20 minutes. The suppressor serum was drained off and the sections or cells were then incubated with the diluted
15   CYT-356 monoclonal antibody for 1 hour. Samples were then washed with PBS and sequentially incubated with secondary antibodies (horse or goat immunoglobulins, 1:200 dilution for 30 minutes), and with avidin-biotin complexes (1:25 dilution for 30 minutes). DAB was used
20   as a chromogen, followed by hematoxylin counterstaining and mounting. Frozen sections of prostate samples and duplicate cell cytospins were used as controls for each experiment. As a positive control, the anti-cytokeratin monoclonal antibody CAM 5.2 was used
25   following the same procedure described above. Tissue sections are considered by us to express the PSM antigen if at least 5% of the cells demonstrate immunoreactivity. The scoring system is as follows: 1 = <5%; 2 = 5-19%; 3 = 20-75%; and 4 = >75% positive
30   cells. Homogeneity versus heterogeneity was accounted for by evaluating positive and negative cells in 3-5 high power light microscopic fields (400x), recording the percentage of positive cells among 100-500 cells. The intensity of immunostaining is graded on a 1+ to 4+
35   scale, where 1+ represents mild, 2-3+ represents moderate, and 4+ represents intense immunostaining as compared to positive controls.

Immunopr cipitation of the PSM Antigen: 80%-confluent LNCaP cells in 100mm petri dishes were starved in RPMI media without methionine for 2 hours, after which ^{35}S-Methionine was added at 100μCi/ml and the cells were

5   grown for another 16-18 hours. Cells were then washed and lysed by the addition of 1ml of lysis buffer (1% Triton X-100, 50mM Hepes pH 7.5, 10% glycerol, 150mM MgCl$_2$, 1mM PMSF, and 1mM EGTA) with incubation for 20 minutes at 4°C. Lysates were pre-cleared by mixing

10   with Pansorbin® cells (Calbiochem®) for 90 minutes at 4°C. Cell lysates were then mixed with Protein A Sepharose® CL-4B beads (Pharmacia®) previously bound with CYT-356 antibody (Cytogen Corp.) and RAM antibody (Accurate Scientific) for 3-4 hours at 4°C. 12μg of

15   antibody was used per 3mg of beads per petri dish. Beads were then washed with HNTG buffer (20mM Hepes pH 7.5, 150mM NaCl, 0.1% Triton X-100, 10% glycerol, and 2mM Sodium Orthovanadate), resuspended in sample loading buffer containing ß-mercaptoethanol, denatured

20   at 95°C for 5-10 minutes and run on a 10% SDS-PAGE gel with a 4° stacking gel at 10 milliamps overnight. Gels were stained with Coomassie Blue, destained with acetic acid/methanol, and dried down in a vacuum dryer at 60°C. Gels were then autoradiographed for 16-24 hours

25   at -70°C (Figures 2A-2D).

Immunoprecipitation and Peptide Sequencing:
The procedure described above for immunoprecipitation was repeated with 8 confluent petri dishes containing

30   approximately 6x10^7 LNCaP cells. The immunoprecipitation product was pooled and loaded into two lanes of a 10% SDS-PAGE gel and electrophoresed at 9-10 milliamps for 16 hours. Proteins were electroblotted onto Nitrocellulose BA-85 membranes

35   (Schleicher and Schuell®) for 2 hours at 75 volts at 4°C in transfer buffer. Membranes were stained with Ponceau Red to visualize the proteins and the 100kD

protein band was excised, solubilized, and digested
proteolytically with trypsin. HPLC was then performed
on the digested sample on an Applied Biosystems Model
171C and clear dominant peptide peaks were selected and
5   sequenced by modified Edman degradation on a modified
post liquid Applied Biosystems Model 477A
Protein/Peptide Microsequencer (23). Sequencing data
on all of the peptides is included within this
document. The amino-terminus of the PSM antigen was
10 sequenced by a similar method which involved purifying
the antigen by immunoprecipitation and transfer via
electro-blotting to a PVDF membrane (Millipore®).
Protein was analyzed on an Applied Biosystems Model
477A Protein/Peptide Sequencer and the amino terminus
15 was found to be <u>blocked</u>, and therefore no sequence data
could be obtained by this technique.

PSM Antigen Peptide Sequences:

20 2T17 #5   SLYES(W)TK (SEQ ID No.)
2T22 #9   (S)YPDGXNLPGG(g)VQR (SEQ ID No.)
2T26 #3   FYDPMFK (SEQ ID No.)
2T27 #4   IYNVIGTL(K) (SEQ ID No.)
2T34 #6   FLYXXTQIPHLAGTEQNFQLAK (SEQ ID No.)
25 2T35 #2   G/PVILYSDPADYFAPD/GVK (SEQ ID No.)
2T38 #1   AFIDPLGLPDRPFYR (SEQ ID No.)
2T46 #8   YAGESFPGIYDALFDIESK (SEQ ID No.)
2T47 #7   TILFAS(W)DAEEFGXX(q)STE(e)A(E)... (SEQ ID No.
)

30

Notes: X means that no residue could be identified at
this position. Capital denotes identification but with
a lower degree of confidence.   (lower case) means
residue present but at very low levels. ... indicates
35 sequence continues but has dropped below detection
limit.

All of these peptide sequences were verified to be unique after a complete homology search of the translated Genbank computer database.

5   **Degenerate PCR:** Sense and anti-sense 5'-unphosphorylated degenerate oligonucleotide primers 17 to 20 nucleotides in length corresponding to portions of the above peptides were synthesized on an Applied Biosystems Model 394A DNA Synthesizer. These primers

10   have degeneracies from 32 to 144. The primers used are shown below. The underlined amino acids in the peptides represent the residues used in primer design.

Peptide 3:   **FYDPMFK** (SEQ ID No.)

15

PSM Primer "A" TT(C or T) - TA(C or T) - GA(C or T) - CCX - ATG - TT (SEQ ID No.)

PSM Primer "B" AAC - ATX - GG(A or G) - TC(A or G) -
20   TA(A or G) - AA (SEQ ID No.   )

Primer A is sense primer and B is anti-sense. Degeneracy is 32-fold.

25   Peptide 4:   **IYNVIGTL(K)** (SEQ ID No. 6)

PSM Primer "C" AT(T or C or A) - TA(T or C) - AA(T or C) - GTX - AT(T or C or A) - GG (SEQ ID No.)

30   PSM Primer "D" CC(A or T or G) - ATX - AC(G or A) - TT(A or G) - TA(A or G or T) - AT (SEQ ID No.)

Primer C is sense primer and D is anti-sense. Degeneracy is 144-fold.

35

Peptide 2:   **G/PVILYSDPADYFAPD/GVK** (SEQ ID No.)

PSM Primer "E"   CCX - GCX - GA(T or C) - TA(T or C) -
TT(T or C) - GC (SEQ ID No.)

PSM Primer "F"   GC(G or A) - AA(A or G) - TA(A or G) -
5   TXC - GCX - GG (SEQ ID No.)

Primer E is sense primer and F is antisense primer.
Degeneracy is 128-fold.

10   Peptide 6:   **FLYXXTQIPHLAGTEQNFQLAK** (SEQ ID No.)

PSM Primer "I"   ACX - GA(A or G) - CA(A or G) - AA(T or
C) - TT(T or C) - CA(A or G) - CT (SEQ ID No.)

15   PSM Primer "J"   AG - (T or C)TG - (A or G)AA - (A or
G)TT - (T or C)TG - (T or C)TC - XGT (SEQ ID No.)

PSM Primer "K"   GA(A or G) - CA(A or G) - AA(T or C) -
TT(T or C) CA(A or G) - CT (SEQ ID No.   )
20
PSM Primer "L"   AG - (T or C)TG - (A or G)AA - (A or
G)TT - (T or C)TG - (T or C)TC (SEQ ID No. 22)

Primers I and K are sense primers and J and L are anti-
25   sense. I and J have degeneracies of 128-fold and K and
L have 32-fold degeneracy.

Peptide 7:   **TILFAS (W) DAEEFGXX (q) STE (e) A (E) ...** (SEQ
ID No.)
30
PSM Primer "M"   TGG - GA(T or C) - GCX - GA(A or G) -
GA(A or G) - TT(C or T) - GG (SEQ ID No.)

PSM Primer "N"   CC - (G or A)AA - (T or C)TC - (T or
35   C)TC - XGC - (A or G)TC - CCA (SEQ ID No.)

PSM Primer "O"   TGG - GA(T or C) - GCX - GA(A or G) -

GA(A or G) - TT (SEQ ID No.)

PSM Primer "P" AA - (T or C)TC - (T or C)TC - XGC - (A or G)TC - CCA (SEQ ID No.)

Primers M and O are sense primers and N and P are anti-sense. M and N have degeneracy of 64-fold and O and P are 32-fold degenerate.

Degenerate PCR was performed using a Perkin-Elmer Model 480 DNA thermal cycler. cDNA template for the PCR was prepared from LNCaP mRNA which had been isolated by standard methods of oligo dT chromatography (Collaborative Research). The cDNA synthesis was carried out as follows:

4.5μl   LNCaP poly A+ RNA (2μg)
1.0μl   Oligo dT primers (0.5μg)
4.5μl   dH$_2$O
10μl

Incubate at 68°C x 10 minutes.
Quick chill on ice x 5 minutes.

Add:

4μl   5 x RT Buffer
2μl   0.1M DTT
1μl   10mM dNTPs
0.5μl   RNasin (Promega)
1.5μl   dH$_2$O
19μl

Incubate for 2 minutes at 37°C.
Add 1μl Superscript® Reverse Transcriptase (Gibco®-BRL)
Incubate for 1 hour at 37°C.

Add 30μl dH$_2$O.
Use 2μl per PCR reaction.

Degenerate PCR reactions were optimized by varying the
annealing temperatures, Mg++ concentrations, primer
concentrations, buffer composition, extension times and
number of cycles. The optimal thermal cycler profile
was: Denaturation at 94°C x 30 seconds, Annealing at
45-55°C for 1 minute (depending on the mean T$_m$ of the
primers used), and Extension at 72°C for 2 minutes.

5μl	10 x PCR Buffer*
5μl	2.5mM dNTP Mix
5μl	Primer Mix (containing 0.5-1.0μg each of
sense	and anti-sense primers)
5μl	100mM ß-mercaptoethanol
2μl	LNCaP cDNA template
5μl	25mM MgCl$_2$ (2.5mM final)
21μl	dH$_2$O
2μl	diluted Taq Polymerase (0.5U/μl)

50μl total volume

Tubes were overlaid with 60μl of light mineral oil and
amplified for 30 cycles. PCR products were analyzed by
electrophoresing 5μl of each sample on a 2-3% agarose
gel followed by staining with Ethidium bromide and
photography.

*10x PCR Buffer
 166mM NH$_4$SO$_4$
 670mM Tris, pH 8.8
 2mg/ml BSA

Representative photographs displaying PCR products are
shown in Figure 5.

Cloning of PCR Products: In order to further analyze

these PCR products, these products were cloned into a
suitable plasmid vector using "TA Cloning" (Invitrogen®
Corp.).   The cloning strategy employed here is to
directly ligate PCR products into a plasmid vector
5   possessing overhanging T residues at the insertion
site, exploiting the fact that Taq polymerase leaves
overhanging A residues at the ends of the PCR products.
The ligation mixes are transformed into competent E.
coli cells and resulting colonies are grown up, plasmid
10   DNA is isolated by the alkaline lysis method (24), and
screened by restriction analysis (Figures 6A-6B).

DNA Sequencing of PCR Products: TA Clones of PCR
products were then sequenced by the dideoxy method (25)
15   using Sequenase (U.S. Biochemical).   3-4μg of each
plasmid DNA was denatured with NaOH and ethanol
precipitated.   Labeling reactions were carried out as
per the manufacturers recommendations using ^{35}S-ATP, and
the reactions were terminated as per the same protocol.
20   Sequencing   products   were   then   analyzed   on   6%
polyacrylamide/7M Urea gels using an IBI sequencing
apparatus.   Gels were run at 120 watts for 2 hours.
Following electrophoresis, the gels were fixed for 15-
20 minutes in 10% methanol/10% acetic acid, transferred
25   onto Whatman 3MM paper and dried down in a Biorad®
vacuum dryer at 80°C for 2 hours.   Gels were then
autoradiographed at room temperature for 16-24 hours.
In order to determine whether the PCR products were the
correct clones, the sequences obtained at the 5' and 3'
30   ends of the molecules were analyzed for the correct
primer sequences, as well as adjacent sequences which
corresponded to portions of the peptides not used in
the design of the primers.

35   IN-20 was confirmed to be correct and represent a
partial cDNA for the PSM gene.   In this PCR reaction,
I and N primers were used.   The DNA sequence reading

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from the I primer was:

ACG GAG CAA AAC TTT CAG CTT GCA AAG (SEQ ID No.   )
 <u>T   E   Q   N   F   Q</u>   L   A   K (SEQ ID No.   )

The underlined amino acids were the portion of peptide
6 that was used to design this sense primer and the
remaining amino acids which agree with those present
within the peptide confirm that this end of the
molecule represents the correct protein (PSM antigen).

When analyzed the other end of the molecule by reading
from the N primer the anti-sense sequence was:

CTC TTC GGC ATC CCA GCT TGC AAA CAA AAT TGT TCT (SEQ ID
No.   )

Sense (complementary) Sequence:

AGA ACA ATT TTG TTT GCA AGC TGG GAT GCC AAG GAG (SEQ ID
No.   )
 R   T   I   L   F   A   S   <u>W   D   A   E   E</u> (SEQ ID
No.   )

The underlined amino acids here represent the portion
of peptide 7 used to create primer N. All of the amino
acids upstream of this primer are correct in the IN-20
clone, agreeing with the amino acids found in peptide
7. Further DNA sequencing has enabled us to identify
the presence of other PSM peptides within the DNA
sequence of the positive clone.

The DNA sequence of this partial cDNA was found to be
unique when screened on the Genbank computer database.

**cDNA Library Construction and Cloning of Full - Length
PSM cDNA:** A cDNA library from LNCaP mRNA was

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constructed using the Superscript® plasmid system (BRL®-Gibco). The library was transformed using competent DH5-α cells and plated onto 100mm plates containing LB plus 100μg/ml of Carbenicillin. Plates

5   were grown overnight at 37°C and colonies were transferred to nitrocellulose filters. Filters were processed and screened as per Grunstein and Hogness (26), using the 1.1kb partial cDNA homologous probe which was radiolabelled with ^{32}P-dCTP by random priming

10   (27). Eight positive colonies were obtained which upon DNA restriction and sequencing analysis proved to represent full-length cDNA molecules coding for the PSM antigen. Shown in Figure 7 is an autoradiogram showing the size of the cDNA molecules represented in the

15   library and in Figure 8 restriction analysis of several full-length clones is shown. Figure 9 is a plasmid Southern analysis of the samples in Figure 8, showing that they all hybridize to the 1.1kb partial cDNA probe.

20

Both the cDNA as well as the antigen have been screened through the Genbank Computer database (Human Genome Project) and have been found to be unique.

25   **Northern Analysis of PSM Gene Expression:** Northern analysis (28) of the PSM gene has revealed that expression is limited to the prostate and to prostate carcinoma.

30   RNA samples (either 10μg of total RNA or 2μg of poly A+ RNA) were denatured and electrophoresed through 1.1% agarose/formaldehyde gels at 60 milliamps for 6-8 hours. RNA was then transferred to Nytran® nylon membranes (Schleicher and Schuell®) by pressure

35   blotting in 10x SSC with a Posi-blotter (Stratagene®). RNA was cross-linked to the membranes using a Stratalinker (Stratagene®) and subsequently baked in a

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vacuum oven at 80°C for 2 hours. Blots were pre-
hybridized at 65°C for 2 hours in prehybridization
solution (BRL®) and subsequently hybridized for 16
hours in hybridization buffer (BRL®) containing 1-2 x

5   10^6 cpm/ml of ^{32} P-labelled random-primed cDNA probe.
Membranes were washed twice in 1x SSPE/1% SDS and twice
in 0.1x SSPE/1% SDS at 42°C. Membranes were then air-
dried and autoradiographed for 12-36 hours at -70°C.

10   **PCR Analysis of PSM Gene Expression in Human Prostate
Tissues:** PCR was performed on 15 human prostate samples
to determine PSM gene expression. Five samples each
from normal prostate tissue, benign prostatic
hyperplasia, and prostate cancer were used (histology

15   confirmed by MSKCC Pathology Department).

10μg of total RNA from each sample was reverse
transcribed to made cDNA template as previously
described in section IV. The primers used corresponded

20   to the 5' and 3' ends of the 1.1kb partial cDNA, IN-20,
and therefore the expected size of the amplified band
is 1.1kb. Since the T_m of the primers is 64°C. PCR
primers were annealed at 60°C. PCR was carried out for
35 cycles using the same conditions previously

25   described in section IV.

LNCaP and H26 - Ras transfected LNCaP (29) were
included as a positive control and DU-145 as a negative
control. 14/15 samples clearly amplified the 1.1kb

30   band and therefore express the gene.

Experimental Results
The gene which encodes the 100kD PSM antigen has been
identified. The complete cDNA sequence is shown in

35   Sequence ID #1. Underneath that nucleic acid sequence
is the predicted translated amino acid sequence. The
total number of the amino acids is 750, ID #2. The

hydrophilicity of the predicted protein sequence is shown in Figures 16:1-11. Shown in Figures 17A-17C are three peptides with the highest point of hydrophilicity. They are: Asp-Glu-Leu-Lys-Ala-Glu (SEQ

5   ID No.); Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No. ; and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.).

By the method of Klein, Kanehisa and DeLisi, a specific membrane-spanning domain is identified. The sequence

10   is from the amino acid #19 to amino acid #44: Ala-Gly-Ala-Leu-Val-Leu-Aal-Gly-Gly-Phe-Phe-Leu-Leu-Gly-Phe-Leu-Phe (SEQ ID No.).

This predicted membrane-spanning domain was computed on

15   PC Gene (computer software program). This data enables prediction of inner and outer membrane domains of the PSM antigen which aids in designing antibodies for uses in targeting and imaging prostate cancer.

20   When the PSM antigen sequence with other known sequences of the GeneBank were compared, homology between the PSM antigen sequence and the transferrin receptor sequence were found. The data are shown in Figure 18.

25

Experimental Discussions

Potential Uses for PSM Antigen:

30   1.   Tumor detection:
Microscopic:
Unambiguous tumor designation can be accomplished by use of probes for different antigens. For prostatic cancer, the PSM antigen probe may prove beneficial.

35   Thus PSM could be used for diagnostic purposes and this could be accomplished at the microscopic level using in-situ hybridization using sense (control) and

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antisense probes derived from the coding region of the cDNA cloned by the applicants. This could be used in assessment of local extraprostatic extension, involvement of lymph node, bone or other metastatic
5   sites. As bone metastasis presents a major problem in prostatic cancer, early detection of metastatic spread is required especially for staging. In some tumors detection of tumor cells in bone marrow portends a grim prognosis and suggests that interventions aimed at
10   metastasis be tried. Detection of PSM antigen expression in bone marrow aspirates or sections may provide such early information. PCR amplification or in-situ hybridization may be used. Using RT-PCR cells in the circulating can be detected by hematogenous
15   metastasis.

         2.   Antigenic site identification
The knowledge of the cDNA for the antigen also provides for the identification of areas that would serve as
20   good antigens for the development of antibodies for use against specific amino acid sequences of the antigen. Such sequences may be at different regions such as outside, membrane or inside of the PSM antigen. The development of these specific antibodies would provide
25   for immunohistochemical identification of the antigen. These derived antibodies could then be developed for use, especially ones that work in paraffin fixed sections as well as frozen section as they have the greatest utility for immunodiagnosis.
30

         3.   Restriction fragment length polymorphism and
              genomic DNA
Restriction fragment length polymorphisms (RFLPS) have proven to be useful in documenting the progression of
35   genetic damage that occurs during tumor initiation and promotion. It may be that RFLP analysis will demonstrate that changes in PSM sequence restriction

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mapping may provide evidence of predisposition to risk
or malignant potential or progression of the prostatic
tumor.

5   Depending on the chromosomal location of the PSM
antigen, the PSM antigen gene may serve as a useful
chromosome location marker for chromosome analysis.

        4.   Serum
10   With the development of antigen specific antibodies, if
the antigen or selected antigen fragments appear in the
serum they may provide for a serum marker for the
presence of metastatic disease and be useful
individually or in combination with other prostate
15   specific markers.

        5.   Imaging
As the cDNA sequence implies that the antigen has the
characteristics of a membrane spanning protein with the
20   majority of the protein on the exofacial surface,
antibodies, especially monoclonal antibodies to the
peptide fragments exposed and specific to the tumor may
provide for tumor imaging local extension of metastatic
tumor or residual tumor following prostatectomy or
25   irradiation.   The knowledge of the coding region
permits the generation of monoclonal antibodies and
these can be used in combination to provide for maximal
imaging purposes.   Because the antigen shares a
similarity with the transferrin receptor based on cDNA
30   analysis (approximately 54%), it may be that there is
a specific normal ligand for this antigen and that
identification of the ligand(s) would provide another
means of imaging.

35   6.   Isolation of ligands
The PSM antigen can be used to isolate the normal
ligand(s) that bind to it. These ligand(s) depending

on specificity may be used for targeting, or their
serum levels may be predictive of disease status. If
it is found that the normal ligand for PSM is a carrier
molecule then it may be that PSM could be used to bind
5   to that ligand for therapy purposes (like an iron
chelating substance) to help remove the ligand from the
circulation. If the ligand promotes tumor growth or
metastasis then providing soluble PSM antigen would
remove the ligand from binding the prostate. Knowledge
10   of PSM antigen structure could lend to generation of
small fragment that binds ligand which could serve the
same purpose.

          7.   Therapeutic uses
15   a) Ligands. The knowledge that the cDNA structure of
PSM antigen shares structural homology with the
transferrin receptor (54% on the nucleic acid level)
implies that there may be an endogenous ligand for the
receptor that may or may not be transferrin-like.
20   Transferrin is thought to be a ligand that transports
iron into the cell after binding to the transferrin
receptor. However, apotransferrin is being reported to
be a growth factor for some cells which express the
transferrin receptor (30). Whether transferrin is a
25   ligand for this antigen or some other ligand binds to
this ligand remains to be determined. If a ligand is
identified it may carry a specific substance such as a
metal ion (iron or zinc or other) into the tumor and
thus serve as a means to deliver toxic substances
30   (radioactive or cytotoxic chemical i.e. toxin like
ricin or cytotoxic alkylating agent or cytotoxic
prodrug) to the tumor.

The main metastatic site for prostatic tumor is the
35   bone.   The bone and bone stroma are rich in
transferrin.   Recent studies suggest that this
microenvironment is what provides the right "soil" for

prostatic metastasis in the bone (31). It may be that this also promotes attachment as well, these factors which reduce this ability may diminish prostatic metastasis to the bone and prostatic metastatic growth
5   in the bone.

It was found that the ligand for the new antigen (thought to be an oncogene and marker of malignant phenotype in breast carcinoma) served to induce
10   differentiation of breast cancer cells and thus could serve as a treatment for rather than promotor of the disease. It may be that ligand binding to the right region of PSM whether with natural ligand or with an antibody may serve a similar function.

15

Antibodies against PSM antigen coupled with a cytotoxic agent will be useful to eliminate prostate cancer cells. Transferrin receptor antibodies with toxin conjugates are cytotoxic to a number of tumor cells as
20   tumor cells tend to express increased levels of transferrin receptor (32). Transferrin receptors take up molecules into the cell by endocytosis. Antibody drug combinations can be toxic. Transferrin linked toxin can be toxic.

25

b)   Antibodies against PSM antigen coupled with a cytotoxic agent will be useful to eliminate prostate cancer cells. The cytotoxic agent may be a radioisotope or toxin as known in ordinary skill of the
30   art. The linkage of the antibody and the toxin or radioisotope can be chemical. Examples of direct linked toxins are doxorubicin, chlorambucil, ricin, pseudomonas exotoxin etc., or a hybrid toxin can be generated ½ with specificity for PSM and the other ½
35   with specificity for the toxin. Such a bivalent molecule can serve to bind to the tumor and the other ½ to deliver a cytotoxic to the tumor or to bind to and

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activate a cytotoxic lymphocyte such as binding to the T_1 - T_3 receptor complex. Antibodies of required specificity can also be cloned into T cells and by replacing the immunoglobulin domain of the T cell

5   receptor (TcR); cloning in the desired MAb heavy and light chains; splicing the U_h and U_L gene segments with the constant regions of the α and ß TCR chains and transfecting these chimeric Ab/TcR genes in the patients' T cells, propagating these hybrid cells and

10   infusing them into the patient (33). Specific knowledge of tissue specific antigens for targets and generation of MAb's specific for such targets will help make this a usable approach. Because the PSM antigen coding region provides knowledge of the entire coding

15   region, it is possible to generate a number of antibodies which could then be used in combination to achieve an additive or synergistic anti-tumor action. The antibodies can be linked to enzymes which can activate non-toxic prodrugs at its site of the tumor

20   such   as   Ab-carboxypeptidase   and   4-(bis(2 chloroethyl)amino)benzoyl-α-glutamic   acid   and   its active parent drug in mice (34).

It is possible to produce a toxic genetic chimera such

25   as TP-40 a genetic recombinant that possesses the cDNA from TGF-alpha and the toxic portion of pseudomonas exotoxin so the TGF and portion of the hybrid binds the epidermal growth factor receptor (EGFR) and the pseudomonas portion gets taken up into the cell

30   enzymatically and inactivates the ribosomes ability to perform protein synthesis resulting in cell death.

In addition, once the ligand for the PSM antigen is identified, toxin can be chemically conjugated to the

35   ligands.   Such   conjugated   ligands   can   be therapeutically useful. Examples of the toxins are daunomycin, chlorambucil, ricin, pseudomonas exotoxin,

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etc. Alternatively, chimeric construct can be created linking the cDNA of the ligand with the cDNA of the toxin. An example of such toxin is TGFα and pseudomonas exotoxin (35).

5

    8.   Others

The PSM antigen may have other uses. It is well known that the prostate is rich in zinc, if the antigen provides function relative to this or other biologic function the PSM antigen may provide for utility in the treatment of other prostatic pathologies such as benign hyperplastic growth and/or prostatitis.

Because purified PSM antigen can be generated, the purified PSM antigen can be linked to beads and use it like a standard "affinity" purification. Serum, urine or other biological samples can be used to incubate with the PSM antigen bound onto beads. The beads may be washed thoroughly and then eluted with salt or pH gradient. The eluted material is SDS gel purified and used as a sample for microsequencing. The sequences will be compared with other known proteins and if unique, the technique of degenerated PCR can be employed for obtaining the ligand. Once known, the affinity of the ligand will be determined by standard protocols (15).

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EXAMPLE 2:

EXPRESSION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN

5   A 2.65 kb complementary DNA encoding PSM was cloned.
Immunohistochemical analysis of the LNCaP, DU-145, and
PC-3 prostate cancer cell lines for PSM expression
using the 7E11-C5.3 antibody reveals intense staining
in the LNCaP cells, with no detectable expression in
10 both the DU-145 and PC-3 cells. Coupled *in-vitro*
transcription/ translation of the 2.65 kb full-length
PSM cDNA yields an 84 kDa protein corresponding to the
predicted polypeptide molecular weight of PSM. Post-
translational modification of this protein with
15 pancreatic canine microsomes yields the expected 100
kDa PSM antigen. Following transfection of PC-3 cells
with the full-length PSM cDNA in a eukaryotic
expression vector applicant's detect expression of the
PSM glycoprotein by Western analysis using the 7E11-
20 C5.3 monoclonal antibody. Ribonuclease protection
analysis demonstrates that the expression of PSM mRNA
is almost entirely prostate-specific in human tissues.
PSM expression appears to be highest in hormone-
deprived states and is hormonally modulated by
25 steroids, with DHT downregulating PSM expression in the
human prostate cancer cell line LNCaP by 8-10 fold,
testosterone downregulating PSM by 3-4 fold, and
corticosteroids showing no significant effect. Normal
and malignant prostatic tissues consistently show high
30 PSM expression, whereas heterogeneous, and at times
absent, from expression of PSM in benign prostatic
hyperplasia. LNCaP tumors implanted and grown both
orthotopically and subcutaneously in nude mice,
abundantly express PSM providing an excellent *in-vivo*
35 model system to study the regulation and modulation of
PSM expression.

Materials and Methods:

Cells and Reagents: The LNCaP, DU-145, and PC-3 cell
lines were obtained from the American Type Culture
5   Collection.   Details regarding the establishment and
characteristics of these cell lines have been
previously published (5A,7A,8A).   Unless specified
otherwise, LNCaP cells were grown in RPMI 1640 media
supplemented with L-glutamine, nonessential amino
10   acids, and 5% fetal calf serum (Gibco-BRL,
Gaithersburg, MD.) in a CO_2 incubator at 37C.   DU-145
and PC-3 cells were grown in minimal essential medium
supplemented with 10% fetal calf serum. All cell media
were obtained from the MSKCC Media Preparation
15   Facility.   Restriction and modifying enzymes were
purchased from Gibco-BRL unless otherwise specified.

Immunohistochemical Detection of PSM: Avidin-biotin
20   method of detection was employed to analyze prostate
cancer cell lines for PSM antigen expression (9A).
Cell cytospins were made on glass slides using $5x10^4$
cells/100ul per slide. Slides were washed twice with
PBS and then incubated with the appropriate suppressor
25   serum for 20 minutes. The suppressor serum was drained
off and the cells were incubated with diluted 7E11-C5.3
(5g/ml) monoclonal antibody for 1 hour. Samples were
then washed with PBS and sequentially incubated with
secondary antibodies for 30 minutes and with avidin-
30   biotin complexes for 30 minutes. Diaminobenzidine
served as the chromogen and color development followed
by hematoxylin counterstaining and mounting. Duplicate
cell cytospins were used as controls for each
experiment.   As a positive control, the anti-
35   cytokeratin monoclonal antibody CAM 5.2 was used
following the same procedure described above. Human EJ
bladder carcinoma cells served as a negative control.

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In-Vitro Transcription/Translation of PSM Antigen:
Plasmid 55A containing the full length 2.65 kb PSM cDNA
in the plasmid pSPORT 1 (Gibco-BRL) was transcribed in-
vitro using the Promega TNT system (Promega Corp.
5   Madison, WI). T7 RNA polymerase was added to the cDNA
in a reaction mixture containing rabbit reticulocyte
lysate, an amino acid mixture lacking methionine,
buffer, and ^{35}S-Methionine (Amersham) and incubated at
30C for 90 minutes. Post-translational modification of
10   the resulting protein was accomplished by the addition
of pancreatic canine microsomes into the reaction
mixture (Promega Corp. Madison, WI.). Protein products
were analyzed by electrophoresis on 10% SDS-PAGE gels
which were subsequently treated with Amplify
15   autoradiography enhancer (Amersham, Arlington Heights,
IL.) according to the manufacturers instructions and
dried at 80C in a vacuum dryer. Gels were
autoradiographed overnight at -70C using Hyperfilm MP
(Amersham).
20

Transfection of PSM into PC-3 Cells: The full length
PSM cDNA was subcloned into the pREP7 eukaryotic
expression vector (Invitrogen, San Diego, CA.).
Plasmid DNA was purified from transformed DH5-alpha
25   bacteria (Gibco-BRL) using Qiagen maxi-prep plasmid
isolation columns (Qiagen Inc., Chatsworth, CA.).
Purified plasmid DNA (6-10g) was diluted with 900ul of
Optimem media (Gibco-BRL) and mixed with 30ul of
Lipofectin reagent (Gibco-BRL) which had been
30   previously diluted with 900l of Optimem media. This
mixture was added to T-75 flasks of 40-50% confluent
PC-3 cells in Optimem media. After 24-36 hours, cells
were trypsinized and split into 100mm dishes
containing RPMI 1640 media supplemented with 10% fetal
35   calf serum and 1 mg/ml of Hygromycin B (Calbiochem, La
Jolla, CA.). The dose of Hygromycin B used was
previously determined by a time course/dose response

cytotoxicity assay. Cells were maintained in this media for 2-3 weeks with changes of media and Hygromycin B every 4-5 days until discrete colonies appeared. Colonies were isolated using 6mm cloning
5   cylinders and expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 plasmid alone. RNA was isolated from the transfected cells and PSM mRNA expression was detected by both RNase Protection analysis (described later) and
10   by Northern analysis.

Western Blot Detection of PSM Expression: Crude protein lysates were isolated from LNCaP, PC-3, and PSM-transfected PC-3 cells as previously described (10A).
15   LNCaP cell membranes were also isolated according to published methods (10A). Protein concentrations were quantitated by the Bradford method using the BioRad protein reagent kit (BioRad, Richmond, CA.). Following denaturation, $20 \mu g$ of protein was electrophoresed on a
20   10% SDS-PAGE gel at 25 mA for 4 hours. Gels were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA.) overnight at 4C. Membranes were blocked in 0.15M NaCl/0.01M Tris-HCl (TS) plus 5% BSA followed by a 1 hour incubation with 7E11-C5.3 monoclonal
25   antibody ($10 \mu g/ml$). Blots were washed 4 times with 0.15M NaCl/0.01M Tris-HCl/0.05% Triton-X 100 (TS-X) and incubated for 1 hour with rabbit anti-mouse IgG (Accurate Scientific, Westbury, N.Y.) at a concentration of $10 \mu g/ml$.
30

Blots were then washed 4 times with TS-X and labeled with ^{125}I-Protein A (Amersham, Arlington Heights, IL.) at a concentration of 1 million cpm/ml. Blots were then washed 4 times with TS-X and dried on Whatman 3MM
35   paper, followed by overnight autoradiography at -70C using Hyperfilm MP (Amersham).

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Orthotopic and Subcutaneous LNCaP Tumor Growth in Nude Mice: LNCaP cells were harvested from sub-confluent cultures by a one minute exposure to a solution of 0.25% trypsin and 0.02% EDTA. Cells were resuspended in RPMI 1640 media with 5% fetal bovine serum, washed and diluted in either Matrigel (Collaborative Biomedical Products, Bedford, MA.) or calcium and magnesium-free Hank's balanced salt solution (HBSS). Only single cell suspensions with greater than 90% viability by trypan blue exclusion were used for in vivo injection. Male athymic Swiss (nu/nu) nude mice 4-6 weeks of age were obtained from the Memorial Sloan-Kettering Cancer Center Animal Facility. For subcutaneous tumor cell injection one million LNCaP cells resuspended in 0.2 mls. of Matrigel were injected into the hindlimb of each mouse using a disposable syringe fitted with a 28 gauge needle. For orthotopic injection, mice were first anesthetized with an intraperitoneal injection of Pentobarbital and placed in the supine position. The abdomen was cleansed with Betadine and the prostate was exposed through a midline incision. 2.5 million LNCaP tumor cells in 0.1 ml. were injected directly into either posterior lobe using a 1 ml disposable syringe and a 28 gauge needle. LNCaP cells with and without Matrigel were injected. Abdominal closure was achieved in one layer using Autoclip wound clips (Clay Adams, Parsippany, N.J.). Tumors were harvested in 6-8 weeks, confirmed histologically by faculty of the Memorial Sloan-Kettering Cancer Center Pathology Department, and frozen in liquid nitrogen for subsequent RNA isolation.

RNA Isolation: Total cellular RNA was isolated from cells and tissues by standard techniques (11,12) as well as by using RNAzol B (Cinna/Biotecx, Houston, TX.). RNA concentrations and quality were assessed by UV spectroscopy on a Beckman DU 640 spectrophotometer

and by gel analysis. Human tissue total RNA samples
were purchased from Clontech Laboratories, Inc., Palo
Alto, CA.

5   **Ribonuclease Protection Assays:** A portion of the PSM
cDNA was subcloned into the plasmid vector pSPORT 1
(Gibco-BRL) and the orientation of the cDNA insert
relative to the flanking T7 and SP6 RNA polymerase
promoters was verified· by restriction analysis.
10   Linearization of this plasmid upstream of the PSM
insert followed by transcription with SP6 RNA
polymerase yields a 400 nucleotide antisense RNA probe,
of which 350 nucleotides should be protected from RNase
digestion by PSM RNA. This probe was used in Figure
15   20. Plasmid IN-20, containing a 1 kb partial PSM cDNA
in the plasmid pCR II (Invitrogen) was also used for
riboprobe synthesis.   IN-20 linearized with Xmn I
(Gibco-BRL) yields a 298 nucleotide anti-sense RNA
probe when transcribed using SP6 RNA polymerase, of
20   which 260 nucleotides should be protected from RNase
digestion by PSM mRNA. This probe was used in Figures
21 and 22. Probes were synthesized using SP6 RNA
polymerase (Gibco-BRL), rNTPs (Gibco-BRL), RNAsin
(Promega), and ^{32}P-rCTP (NEN, Wilmington, DE.) according
25   to published protocols (13). Probes were purified over
NENSORB 20 purification columns (NEN) and approximately
1 million cpm of purified, radiolabeled PSM probe was
mixed with 10μ of each RNA and hybridized overnight at
45C using buffers and reagents from the RPA II kit
30   (Ambion, Austin, TX). Samples were processed as per
manufacturer's instructions and analyzed on 5%
polyacrilamide/7M urea denaturing gels using Seq ACRYL
reagents (ISS, Natick, MA.). Gels were pre-heated to
55C and run for approximately 1-2 hours at 25 watts.
35   Gels were then fixed for 30 minutes in 10% methanol/10%
acetic acid, dried onto Whatman 3MM paper at 80C in a
BioRad vacuum dryer and autoradiographed overnight with

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Hyperfilm MP (Amersham). Quantitation of PSM expression was determined by using a scanning laser densitometer (LKB, Piscataway, NJ.).

5   **Steroid Modulation Experiment:** LNCaP cells (2 million) were plated onto T-75 flasks in RPMI 1640 media supplemented with 5% fetal calf serum and grown 24 hours until approximately 30-40% confluent. Flasks were then washed several times with phophate-buffered

10   saline and RPMI medium supplemented with 5% charcoal-extracted serum was added. Cells were then grown for another 24 hours, at which time dihydrotesterone, testosterone, estradiol, progesterone, and dexamethasone (Steraloids Inc., Wilton, NH.) were added

15   at a final concentration of 2 nM. Cells were grown for another 24 hours and RNA was then harvested as previously described and PSM expression analyzed by ribonuclease protection analysis.

20

Experimental Results

      Immunohistochemical Detection of PSM: Using the 7E11-C5.3 anti-PSM monoclonal antibody, PSM expression is

25   clearly detectable in the LNCaP prostate cancer cell line, but not in the PC-3 and DU-145 cell lines (Figures 17A-17C). All normal and malignant prostatic tissues analyzed stained positively for PSM expression.

30   **In-Vitro Transcription/Translation of PSM Antigen:** As shown in Figure 18, coupled in-vitro transcription/ translation of the 2.65 kb full-length PSM cDNA yields an 84 kDa protein species in agreement with the expected protein product from the 750 amino acid PSM

35   open reading frame. Following post-translational modification using pancreatic canine microsomes were obtained a 100 kDa glycosylated protein species

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consistent with the mature, native PSM antigen.

Detection of PSM Antigen in LNCaP Cell Membranes and Transfected PC-3 Cells: PC-3 cells transfected with the
5   full length PSM cDNA in the pREP7 expression vector were assayed for expression of SM mRNA by Northern analysis. A clone with high PSM mRNA expression was selected for PSM antigen analysis by Western blotting using the 7E11-C5.3 antibody. In Figure 19, the 100
10 kDa PSM antigen is well expressed in LNCaP cell lysate and membrane fractions, as well as in PSM-transfected PC-3 cells but not in native PC-3 cells.   This detectable expression in the transfected PC-3 cells proves that the previously cloned 2.65 kb PSM cDNA
15 encodes the antigen recognized by the 7E11-C5.3 anti-prostate monoclonal antibody.

PSM mRNA Expression: Expression of PSM mRNA in normal human tissues was analyzed using ribonuclease
20 protection assays. Tissue expression of PSM appears predominantly within the prostate, with very low levels of expression detectable in human brain and salivary gland (Figure 20). No detectable PSM mRNA expression was evident in non-prostatic human tissues when
25 analyzed by Northern analysis. On occasion it is noted that detectable PSM expression in normal human small intestine tissue, however this mRNA expression is variable depending upon the specific riboprobe used. All samples of normal human prostate and human
30 prostatic adenocarcinoma assayed have revealed clearly detectable PSM expression, whereas generally decreased or absent expression of PSM in tissues exhibiting benign hyperplasia (Figure 21). In human LNCaP tumors grown both orthotopically and subcutaneously in nude
35 mice abundant PSM expression with or without the use of matrigel, which is required for the growth of subcutaneously implanted LNCaP cells was detected

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(Figure 21).   PSM mRNA expression is distinctly
modulated by the presence of steroids in physiologic
doses (Figure 22). DHT downregulated expression by 8-
10 fold after 24 hours and testosterone diminished PSM
5   expression by 3-4 fold.   Estradiol and progesterone
also downregulated PSM expression in LNCaP cells,
perhaps as a result of binding to the mutated androgen
receptor known to exist in the LNCaP cell. Overall,
PSM expression is highest in the untreated LNCaP cells
10   grown in steroid-depleted media, a situation that
simulates the hormone-deprived (castrate) state in-
vivo. This experiment was repeated at steroid dosages
ranging from 2-200 nM and at time points from 6 hours
to 7 days with similar results; maximal downregulation
15   of PSM mRNA was seen with DHT at 24 hours at doses of
2-20 nM.

Experimental Discussion

20   Previous research has provided two valuable prostatic
bio-markers, PAP and PSA, both of which have had a
significant impact on the diagnosis, treatment, and
management of prostate malignancies. The present work
describing the preliminary characterization of the
25   prostate-specific membrane antigen (PSM) reveals it to
be a gene with many interesting features.   PSM is
almost entirely prostate-specific as are PAP and PSA,
and as such may enable further delineation of the
unique functions and behavior of the prostate.   The
30   predicted sequence of the PSM protein (3) and its
presence in the LNCaP cell membrane as determined by
Western blotting and immunohistochemistry, indicate
that it is an integral membrane protein. Thus, PSM
provides an attractive cell surface epitope for
35   antibody-directed diagnostic imaging and cytotoxic
targeting modalities (14). The ability to synthesize
the PSM antigen in-vitro and to produce tumor

xenografts maintaining high levels of PSM expression provides us with a convenient and attractive model system to further study and characterize the regulation and modulation of PSM expression. Also, the high level of PSM expression in the LNCaP cells provides an excellent *in-vitro* model system. Since PSM expression is hormonally-responsive to steroids and may be highly expressed in hormone-refractory disease (15). The detection of PSM mRNA expression in minute quantities in brain, salivary gland, and small intestine warrants further investigation, although these tissues were negative for expression of PSM antigen by immunohistochemistry using the 7E11-C5.3 antibody (16). In all of these tissues, particularly small intestine, mRNA expression using a probe corresponding to a region of the PSM cDNA near the 3' end, whereas expression when using a 5' end PSM probe was not detected. These results may indicate that the PSM mRNA transcript undergoes alternative splicing in different tissues.

Applicants approach is based on prostate tissue specific promotor: enzyme or cytokine chimeras. Promotor specific activation of prodrugs such as non toxic gancyclovir which is converted to a toxic metabolite by herpes simplex thymidine kinase or the prodrug 4-(bis(2chloroethyl)amino)benzoyl-1-glutamic acid to the benzoic acid mustard alkylating agent by the pseudomonas carboxy peptidase G2 was examined. As these drugs are activated by the enzyme (chimera) specifically in the tumor the active drug is released only locally in the tumor environment, destroying the surrounding tumor cells. Promotor specific activation of cytokines such as IL-12, IL-2 or GM-CSF for activation and specific antitumor vaccination is examined. Lastly the tissue specific promotor activation of cellular death genes may also prove to be useful in this area.

Gene Th rapy Chimeras: The establishment of "chimeric DNA" for gene therapy requires the joining of different segments of DNA together to make a new DNA that has characteristics of both precursor DNA species involved
5   in the linkage. In this proposal the two pieces being linked involve different functional aspects of DNA, the promotor region which allows for the reading of the DNA for the formation of mRNA will provide specificity and the DNA sequence coding for the mRNA will provide for
10   therapeutic functional DNA.

DNA-Specified Enzyme or Cytokine mRNA: When effective, antitumor drugs can cause the regression of very large amounts of tumor. The main requirements for antitumor
15   drug activity is the requirement to achieve both a long enough time (t) and high enough concentration (c) (cxt) of exposure of the tumor to the toxic drug to assure sufficient cell damage for cell death to occur. The drug also must be "active" and the toxicity for the
20   tumor greater than for the hosts normal cells (22). The availability of the drug to the tumor depends on tumor blood flow and the drugs diffusion ability. Blood flow to the tumor does not provide for selectivity as blood flow to many normal tissues is
25   often as great or greater than that to the tumor. The majority of chemotherapeutic cytotoxic drugs are often as toxic to normal tissue as to tumor tissue. Dividing cells are often more sensitive than non-dividing normal cells, but in many slow growing solid tumors such as
30   prostatic cancer this does not provide for antitumor specificity (22).

Previously a means to increase tumor specificity of antitumor drugs was to utilize tumor associated enzymes
35   to activate nontoxic prodrugs to cytotoxic agents (19). A problem with this approach was that most of the enzymes found in tumors were not totally specific in

their activity and similar substrate active enzymes or
the same enzyme at only slightly lower amounts was
found in other tissue and thus normal tissues were
still at risk for damage.

To provide absolute specificity and unique activity,
viral, bacterial and fungal enzymes which have unique
specificity for selected prodrugs were found which were
not present in human or other animal cells. Attempts
to utilize enzymes such as herpes simplex thymidine
kinase,   bacterial   cytosine   deaminase   and
carboxypeptidase G-2 were linked to antibody targeting
systems with modest success (19).   Unfortunately,
antibody targeted enzymes limit the number of enzymes
available per cell. Also, most antibodies do not have
a high tumor target to normal tissue ratio thus normal
tissues are still exposed reducing the specificity of
these unique enzymes. Antibodies are large molecules
that have poor diffusion properties and the addition of
the enzymes molecular weight further reduces the
antibodies diffusion.

Gene therapy could produce the best desired result if
it could achieve the specific expression of a protein
in the tumor and not normal tissue in order that a high
local concentration of the enzyme be available for the
production in the tumor environment of active drug
(21).

Cytokines:
Results demonstrated that tumors such as the bladder
and prostate were not immunogenic, that is the
administration of irradiated tumor cells to the animal
prior to subsequent administration of non-irradiated
tumor cells did not result in a reduction of either the
number of tumor cells to produce a tumor nor did it
reduce the growth rate of the tumor. But if the tumor

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was transfected with a retrovirus and secreted large
concentrations of cytokines such as Il-2 then this
could act as an antitumor vaccine and could also reduce
the growth potential of an already established and
5   growing tumor.   IL-2 was the best, GM-CSF also had
activity whereas a number of other cytokines were much
less active.   In clinical studies just using IL-2 for
immunostimulation, very large concentrations had to be
given which proved to be toxic.   The key to the success
10   of the cytokine gene modified tumor cell is that the
cytokine is produced at the tumor site locally and is
not toxic and that it stimulates immune recognition of
the tumor and allows specific and non toxic recognition
and destruction of the tumor.   The exact mechanisms of
15   how IL-2 production by the tumor cell activates immune
recognition is not fully understood, but one
explanation is that it bypasses the need for cytokine
production by helper T cells and directly stimulates
tumor   antigen   activated   cytotoxic   CD8   cells.
20   Activation of antigen presenting cells may also occur.

Tissue Promotor-Specific Chimera DNA Activation

Non-Prostatic Tumor Systems:
25   It has been observed in non-prostatic tumors that the
use of promotor specific activation can selectively
lead to tissue specific gene expression of the
transfected gene.   In melanoma the use of the
tyrosinase promotor which codes for the enzyme
30   responsible for melanin expression produced over a 50
fold greater expression of the promotor driven reporter
gene expression in melanoma cells and not non melanoma
cells.   Similar specific activation was seen in the
melanoma cells transfected when they were growing in
35   mice.   In that experiment no non-melanoma or melanocyte
cell expressed the tyrosinase drive reporter gene
product.   The research group at Welcome Laboratories

have cloned and sequenced the promoter region of the gene coding for carcinoembryonic antigen (CEA). CEA is expressed on colon and colon carcinoma cells but specifically on metastatic. A gene chimera was generated which cytosine deaminase. Cytosine deaminase which converts 5 flurorocytosine into 5 fluorouracil and observed a large increase in the ability to selectively kill CEA promotor driven colon tumor cells but not normal liver cells. In vivo they observed that bystander tumor cells which were not transfected with the cytosine deaminase gene were also killed, and that there was no toxicity to the host animal as the large tumors were regressing following treatment. Herpes simplex virus, (HSV), thymidine kinase similarly activates the prodrug gancyclovir to be toxic towards dividing cancer cells and HSV thymidine kinase has been shown to be specifically activatable by tissue specific promoters.

Prostatic Tumor Systems: The therapeutic key to effective cancer therapy is to achieve specificity and spare the patient toxicity. Gene therapy may provide a key part to specificity in that non-essential tissues such as the prostate and prostatic tumors produce tissue specific proteins, such as acid phosphatase (PAP), prostate specific antigen (PSA), and a gene which was cloned, prostate-specific membrane antigen (PSM). Tissues such as the prostate contain selected tissue specific transcription factors which are responsible for binding to the promoter region of the DNA of these tissue specific mRNA. The promoter for PSA has been cloned. Usually patients who are being treated for metastatic prostatic cancer have been put on androgen deprivation therapy which dramatically reduces the expression of mRNA for PSA. PSM on the other hand increases in expression with hormone deprivation which-means it would be even more intensely

expressed on patients being treated with hormone therapy.

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EXAMPLE 3:

**Sensitive   D tection   of   Prostatic   Hematogenous
Micrometastases Using PSA and PSM-Derived Primers in
the Polymerase Chain Reaction**

A PCR-based assay was developed enabling sensitive
detection of hematogenous micrometastases in patients
with prostate cancer. "Nested PCR", was performed by
amplifying mRNA sequences unique to prostate-specific
antigen and to the prostate-specific membrane antigen,
and   have   compared   their   respective   results.
Micrometastases were detected in 2/30 patients (6.7%)
by PCR with PSA-derived primers, while PSM-derived
primers detected tumor cells in 19/16 patients (63.3%).
All 8 negative controls were negative with both PSA and
PSM PCR. Assays were repeated to confirm results, and
PCR products were verified by DNA sequencing and
Southern   analysis.   Patients   harboring   circulating
prostatic tumor cells as detected by PSM, and not by
PSA-PCR included 4 patients previously treated with
radical prostatectomy and with non-measurable serum PSA
levels at the time of this assay. The significance of
these   findings   with   respect   to   future   disease
recurrence and progression will be investigated.

Improvement in the overall survival of patients with
prostate cancer will depend upon earlier diagnosis.
Localized disease, without evidence of extra-prostatic
spread, is successfully treated with either radical
prostatectomy   or   external   beam   radiation,   with
excellent long-term results (2,3). The major problem
is that approximately two-thirds of men diagnosed with
prostate cancer already have evidence of advanced
extra-prostatic spread at the time of diagnosis, for
which there is at present no cure (4).   The use of
clinical   serum   markers   such   as   prostate-specific

antigen (PSA) and prostatic acid phosphatase (PAP) have enabled clinicians to detect prostatic carcinomas earlier and provide useful parameters to follow responses to therapy (5). Yet, despite the advent of sensitive serum PSA assays, radionuclide bone scans, CT scans and other imaging modalities, results have not detected the presence of micrometastatic cells prior to their establishment of solid metastases. Previous work has been done utilizing the polymerase chain reaction to amplify mRNA sequences unique to breast, leukemia, and other malignant cells in the circulation and enable early detection of micrometastases (6,7). Recently, a PCR-based approach utilizing primers derived from the PSA DNA sequence was published (8). In this study 3/12 patients with advanced, stage D prostate cancer had detectable hematogenous micrometastases.

PSM appears to be an integral membrane glycoprotein which is very highly expressed in prostatic tumors and metastases and is almost entirely prostate-specific (10). Many anaplastic tumors and bone metastases have variable and at times no detectable expression of PSA, whereas these lesions appear to consistently express high levels of PSM. Prostatic tumor cells that escape from the prostate gland and enter the circulation are likely to have the potential to form metastases and are possibly the more aggressive and possibly anaplastic cells, a population of cells that may not express high levels of PSA, but may retain high expression of PSM. DNA primers derived from the sequences of both PSA and PSM in a PCR assay were used to detect micrometastatic cells in the peripheral circulation. Despite the high level of amplification and sensitivity of conventional RNA PCR, "Nested" PCR approach in which a amplified target sequence was employed, and subsequently use this PCR product as the template for another round of PCR amplification with a new set of primers totally

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contained within the sequence of the previous product. This approach has enabled us to increase the level of detection from one prostatic tumor cell per 10,000 cells to better than one cell per ten million cells.

Materials and Methods

Cells and Reagents: LNCaP and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD.). Details regarding the establishment and characteristics of these cell lines have been previously published (11,12). Cells were grown in RPMI 1640 media supplemented with L-glutamine, nonessential amino acids, obtained from the MSKCC Media Preparation Facility, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD.) in a CO_2 incubator at 37C. All cell media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were of the highest grade possible and were obtained from Sigma Chemical Company, St. Louis, MO.

Patient Blood Specimens: All blood specimens used in this study were from patients seen in the outpatient offices of urologists on staff at MSKCC. Two anti-coagulated (purple top) tubes per patient were obtained at the time of their regularly scheduled blood draws. Specimen procurement was conducted as per the approval of the MSKCC Institutional Review Board. Samples were promptly brought to the laboratory for immediate processing. Serum PSA and PAP determinations were performed by standard techniques by the MSKCC Clinical Chemistry Laboratory. PSA determinations were performed using the Tandem PSA assay (Hybritech, San Diego, CA.). The eight blood specimens used as negative controls were from 2 males with normal serum PSA values and biopsy-proven BPH, one healthy female, 3 healthy males, one patient with bladder cancer, and

one patient with acute promyelocytic leukemia.

Blood Sample Processing/RNA Extraction: 4 ml of whole
anticoagulated venous blood was mixed with 3 ml of ice
5   cold phosphate buffered saline and then carefully
layered atop 8 ml of Ficoll (Pharmacia, Uppsala,
Sweden) in a 15-ml polystyrene tube. Tubes were
centrifuged at 200 x g for 30 min. at 4C. Using a
sterile pasteur pipette, the buffy coat layer (approx.
10   1 ml.) was carefully removed and rediluted up to 50 ml
with ice cold phosphate buffered saline in a 50 ml
polypropylene tube. This tube was then centrifuged at
2000 x g for 30 min at 4C. The supernatant was
carefully decanted and the pellet was allowed to drip
15   dry. One ml of RNazol B was then added to the pellet
and total RNA was isolated as per manufacturers
directions (Cinna/Biotecx, Houston, TX.). RNA
concentrations and purity were determined by UV
spectroscopy on a Beckman DU 640 spectrophotometer and
20   by gel analysis.

Determination of PCR Sensitivity: RNA was isolated from
LNCaP cells and from mixtures of LNCaP and MCF-7 cells
at fixed ratios (i.e. 1:100, 1:1000, etc.) using RNAzol
25   B. Nested PCR was then performed as described below
with both PSA and PSM primers in order to determine the
limit of detection for the assay. LNCaP:MCF-7
(1:100,000) cDNA was diluted with distilled water to
obtain concentrations of 1:1,000,000 and 1:10,000,000.
30   MCF-7 cells were chosen because they have been
previously tested and shown not to express PSM by PCR.

Polymerase Chain Reaction: The PSA outer primers used
span portions of exons 4 and 5 to yield a 486 bp PCR
35   product and enable differentiation between cDNA and
possible contaminating genomic DNA amplification. The
upstream primer sequence beginning at nucleotide 494 in

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PSA cDNA sequence is 5'-TACCCACTGCATCAGGAACA-3' (SEQ. ID. No.) and the downstream primer at nucleotide 960 is 5'-CCTTGAAGCACACCATTACA-3' (SEQ. ID. No.). The PSA inner upstream primer (beginning at nucleotide 559)
5   5'-ACACAGGCCAGGTATTTCAG-3' (SEQ. ID. No.) and the downstream primer (at nucleotide 894) 5'-GTCCAGCGTCCAGCACACAG-3' (SEQ. ID. No.) yield a 355 bp PCR product. All primers were synthesized by the MSKCC Microchemistry Core Facility. 5μg of total RNA was
10   reverse-transcribed into cDNA in a total volume of 20μl using Superscript reverse transcriptase (Gibco-BRL) according to the manufacturers recommendations. 1μl of this cDNA served as the starting template for the outer primer PCR reaction. The 20μl PCR mix included: 0.5U
15   Taq polymerase (Promega Corp., Madison, WI.), Promega reaction buffer, 1.5mM $MgCl_2$, 200mM dNTPs, and 1.0μM of each primer. This mix was then transferred to a Perkin Elmer 9600 DNA thermal cycler and incubated for 25 cycles. The PCR profile was as follows: 94C x 15
20   sec., 60C x 15 sec., and 72C for 45 sec. After 25 cycles, samples were placed on ice, and 1μl of this reaction mix served as the template for another round of PCR using the inner primers. The first set of tubes were returned to the thermal cycler for 25 additional
25   cycles. PSM-PCR required the selection of primer pairs that also spanned an intron in order to be certain that cDNA and not genomic DNA were being amplified.

The PSM outer primers yield a 946 bp product and the
30   inner primers a 434 bp product. The PSM outer upstream primer used was 5'-ATGGGTGTTTGGTGGTATTGACC-3' (SEQ. ID. No.) (beginning at nucleotide 1401) and the downstream primer (at nucleotide 2348) was 5'-TGCTTGGAGCATAGATGACATGC-3' (SEQ. ID. No.) The PSM
35   inner upstream primer (at nucleotide 1581) was 5'-ACTCCTTCAAGAGCGTGGCG-3' (SEQ. ID. No.) and the downstream primer (at nucleotide 2015) was 5'-

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AACACCATCCCTCCTCGAACC-3' (SEQ. ID. No.). cDNA used
was the same as for the PSA assay. The 50l PCR mix
included: 1U Taq Polymerase (Promega), 250M dNTPs, 10mM
-mercaptoethanol, 2mM MgCl$_2$, and 5l of a 10x buffer mix

5 containing: 166mM NH$_4$SO$_4$, 670mM Tris pH 8.8, and 2 mg/ml
of acetylated BSA. PCR was carried out in a Perkin
Elmer 480 DNA thermal cycler with the following
parameters: 94C x 4 minutes for 1 cycle, 94C x 30 sec.,
58C x 1 minute, and 72C x 1 minute for 25 cycles,

10 followed by 72C x 10 minutes. Samples were then iced
and 2l of this reaction mix was used as the template
for another 25 cycles with a new reaction mix
containing the inner PSM primers. cDNA quality was
verified by performing control reactions using primers

15 derived from -actin yielding a 446 bp PCR product. The
upstream primer used was 5'-AGGCCAACCGCGAGAAGATGA-3'
(SEQ. ID. No.) (exon 3) and the downstream primer was
5'-ATGTCACACTGGGGAAGC-3' (SEQ. ID. No.) (exon 4).
The entire PSA mix and 10l of each PSM reaction mix

20 were run on 1.5-2% agarose gels, stained with ethidium
bromide and photographed in an Eagle Eye Video Imaging
System (Stratagene, Torrey Pines, CA.). Assays were
repeated at least 3 times to verify results.

25 **Cloning and Sequencing of PCR Products:** PCR products
were cloned into the pCR II plasmid vector using the TA
cloning system (Invitrogen). These plasmids were
transformed into competent E. coli cells using standard
methods (13) and plasmid DNA was isolated using Magic

30 Minipreps (Promega) and screened by restriction
analysis. TA clones were then sequenced by the dideoxy
method (14) using Sequenase (U.S. Biochemical). 3-4g
of each plasmid was denatured with NaOH and ethanol
precipitated. Labeling reactions were carried out

35 according to the manufacturers recommendations using
[35]S-dATP (NEN), and the reactions were terminated as
discussed in the same protocol. Sequencing products

were then analyzed on 6% polyacrilamide/7M urea gels run at 120 watts for 2 hours. Gels were fixed for 20 minutes in 10% methanol/10% acetic acid, transferred to Whatman 3MM paper and dried down in a vacuum dryer for

5   2 hours at 80C. Gels were then autoradiographed at room temperature for 18 hours.

Southern Analysis: Ethidium-stained agarose gels of PCR products were soaked for 15 minutes in 0.2N HCl,

10   followed by 30 minutes each in 0.5N NaOH/1.5M NaCl and 0.1M Tris pH 7.5/1.5M NaCl. Gels were then equilibrated for 10 minutes in 10x SSC (1.5M NaCl/0.15M Sodium Citrate. DNA was transferred onto Nytran nylon membranes (Schleicher and Schuell) by pressure

15   blotting in 10x SSC with a Posi-blotter (Stratagene). DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Blots were pre-hybridized at 65C for 2 hourthes and subsequently hybridized with denatured ^{32}P-labeled, random-primed cDNA probes (either

20   PSM or PSA)(9,15). Blots were washed twice in 1x SSPE/0.5% SDS at 42C and twice in 0.1x SSPE/0.5% SDS at 50C for 20 minutes each. Membranes were air-dried and autoradiographed for 30 minutes to 1 hour at -70C with Kodak X-Omat film.

25

Experimental Results

PCR amplification with nested primers improved the level of detection of prostatic cells from

30   approximately one prostatic cell per 10,000 MCF-7 cells to better than one cell per million MCF-7 cells, using either PSA or PSM-derived primers (Figures 26 and 27). This represents a substantial improvement in the ability to detect minimal disease. Characteristics of

35   the 16 patients analyzed with respect to their clinical stage, treatment, serum PSA and PAP values, and results of the assay are shown. In total, PSA-PCR detected

tumor cells in 2/30 patients (6.7%), whereas PSM-PCR detected cells in 19/30 patients (63.3%). There were no patients positive for tumor cells by PSA and not by PSM, while PSM provided 8 positive patients not detected by PSA. Patients 10 and 11 in table 1, both with very advanced hormone-refractory disease were detected by both PSA and PSM. Both of these patients have died since the time these samples were obtained. Patients 4, 7, and 12, all of whom were treated with radical prostatectomies for clinically localized disease, and all of whom have non-measurable serum PSA values 1-2 years postoperatively were positive for circulating prostatic tumor cells by PSM-PCR, but negative by PSA-PCR. A representative ethidium stained gel photograph for PSM-PCR is shown in Figure 28. Samples run in lane A represent PCR products generated from the outer primers and samples in lanes labeled B are products of inner primer pairs. The corresponding PSM Southern blot autoradiograph is shown in Figure 29. The sensitivity of the Southern blot analysis exceeded that of ethidium staining, as can be seen in several samples where the outer product is not visible on Figure 28, but is detectable by Southern blotting as shown in Figure 29. In addition, sample 3 on Figures 28 and 29 (patient 6 in Figure 30) appears to contain both outer and inner bands that are smaller than the corresponding bands in the other patients. DNA sequencing has confirmed that the nucleotide sequence of these bands matches that of PSM, with the exception of a small deletion. This may represent either an artifact of PCR, alternative splicing of PSM mRNA in this patient, or a PSM mutation. All samples sequenced and analyzed by Southern analysis have been confirmed as true positives for PSA and PSM.

Experimental Details

The ability to accurately stage patients with prostate

cancer at the time of diagnosis is clearly of paramount
importance in selecting appropriate therapy and in
predicting long-term response to treatment, and
potential cure.   Pre-surgical staging presently
5   consists of physical examination, serum PSA and PAP
determinations, and numerous imaging modalities
including transrectal ultrasonography, CT scanning,
radionuclide bone scans, and even MRI scanning.   No
present modality, however, addresses the issue of
10   hematogenous micrometastatic disease and the potential
negative impact on prognosis that this may produce.
Previous work has shown that only a fractional
percentage of circulating tumor cells will inevitably
go on to form a solid metastasis (16), however, the
15   detection of and potential quantification of
circulating tumor cell burden may prove valuable in
more accurately staging disease.   The long-term impact
of hematogenous micrometastatic disease must be studied
by comparing the clinical courses of patients found to
20   have these cells in their circulation with patients of
similar stage and treatment who test negatively.

The significantly higher level of detection of tumor
cells with PSM as compared to PSA is not surprising to
25   us, since more consistent expression of PSM in prostate
carcinomas of all stages and grades as compared to
variable expression of PSA in more poorly
differentiated and anaplastic prostate cancers is
noted.   The detection of tumor cells in the three
30   patients that had undergone radical prostatectomies
with subsequent undetectable amounts of serum PSA was
suprising.   These patients would be considered to be
surgical "cures" by standard criteria, yet they
apparently continue to harbor prostatic tumor cells.
35   It will be interesting to follow the clinical course of
these patients as compared to others without PCR
evidence of residual disease.

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EXAMPLE 4:

**EXPRESSION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN
(PSM) DIMINISHES THE MITOGENIC STIMULATION OF
AGGRESSIVE HUMAN PROSTATIC CARCINOMA CELLS BY
TRANSFERRIN**

An association between transferrin and human prostate
cancer has been suggested by several investigators. It
has been shown that the expressed prostatic secretions
of patients with prostate cancer are enriched with
respect to their content of transferrin and that
prostate cancer cells are rich in transferrin receptors
(J. Urol. 143, 381, 1990). Transferrin derived from
bone marrow has been shown to selectively stimulate the
growth of aggressive prostate cancer cells (PNAS 89,
6197, 1992). DNA sequence analysis has revealed that
a portion of the coding region, from nucleotide 1250 to
1700 possesses a 54% homology to the human transferrin
receptor. PC-3 cells do not express PSM mRNA or
protein and exhibit increased cell growth in response
to transferrin, whereas, LNCaP prostate cancer cells
which highly express PSM have a very weak response to
transferrin. To determine whether PSM expression by
prostatic cancer cells impacts upon their mitogenic
response to transferrin the full-length PSM cDNA was
transfected into the PC-3 prostate cancer cells.
Clones highly expressing PSM mRNA were identified by
Northern analysis and expression of PSM protein was
verified by Western analysis using the anti-PSM
monoclonal antibody 7E11-C5.3.

2×10^4 PC-3 or PSM-transfected PC-3 cells per well ere
plated in RPMI medium supplemented with 10% fetal
bovine serum and at 24 hrs. added 1 μg per ml. of
holotransferrin to the cells. Cells were counted at 1
day to be highly mitogenic to the PC-3 cells. Cells

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were counted at 1 day to determine plating efficiency
and at 5 days to determine the effect of the
transferrin. Experiments were repeated to verify the
results.

5

PC-3 cells experienced an average increase of 275% over
controls, whereas the LNCaP cells were only stimulated
43%. Growth kinetics revealed that the PSM-transfected
PC-3 cells grew 30% slower than native PC-3 cells.
10 This data suggests that PSM expression in aggressive,
metastatic human prostate cancer cells significantly
abrogates their mitogenic response to transferrin.

The use of therapeutic vaccines consisting of cytokine-
15 secreting tumor cell preparations for the treatment of
established prostate cancer was investigated in the
Dunning R3327-MatLyLu rat prostatic adenocarcinoma
model. Only IL-2 secreting, irradiated tumor cell
preparations were capable of curing animals from
20 subcutaneously established tumors, and engendered
immunological memory that protected the animals from
another tumor challenge. Immunotherapy was less
effective when tumors were induced orthotopically, but
nevertheless led to improved outcome, significantly
25 delaying, and occasionally preventing recurrence of
tumors after resection of the cancerous prostate.
Induction of a potent immune response in tumor bearing
animals against the nonimmunogenic MatLyLu tumor
supports the view that active immunotherapy of prostate
30 cancer may have therapeutic benefits.

35

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EXAMPLE 5:

CLONING AND CHARACTERIZATION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) PROMOTER.

5

The expression and regulation of the PSM gene is complex. By immunostaining, PSM antigen was found to be expressed brilliantly in metastasized tumor, and in
10   organ confined tumor, less so in normal prostatic tissue and more heterogenous in BPH. PSM is strongly expressed in both anaplastic and hormone refractory tumors. PSM mRNA has been shown to be down regulated by androgen. Expression of PSM RNA is also modulated
15   by a host of cytokines and growth factors. Knowledge of the regulation of PSM expression should aid in such diagnostic   and   therapeutic   strategies   as imunoscintigraphic imaging of prostate cancer and protate-specific promoter-driven gene therapy.
20

Sequencing of a 3 kb genomic DNA clone that contained 2.5 kb upstream of the transcription start site revealed that two stretches of about 300 b.p. (-260 to -600; and -1325 to -1625) have substantial homology
25   (79-87%) to known genes. The promoter lacks a GC rich region, nor does it have a consensus TATA box. However, it contains a TA-rich region from position -35 to -65.

30   Several consensus recognition sites for general transcription factors such as AP1, AP2, NFkB, GRE and E2-RE were identified. Chimeric constructs containing fragments of the upstream region of the PSM gene fused to a promoterless chloramphenicol acetyl transferase
35   gene were transfected into, and transiently expressed in LNCaP, PC-3, and SW620 (a colonic cell line). With an additional SV40 enhancer, sequence from -565 to +76

exhibited promoter activity in LNCaP but not in PC-3 nor in SW620.

Materials and Methods

5

Cell Lines.   LNCaP and PC-3 prostatic carcinoma cell lines (American Type Culture Collection) were cultured in RPMI and MEM respectively, supplemented with 5% fetal calf serum at 37°C and 5% CO_2. SW620, a colonic

10   cell line, is a gift from Melisa.

Polymerase Chain Reaction. The reaction was performed in a 50 μl volume with a final concentration of the following reagents: 16.6 mM NH_4SO_4, 67 mM Tris-HCl pH

15   8.8, acetylated BSA 0.2 mg/ml, 2mM $MgCl_2$, 250μM dNTPs, 10 mM ß-mercaptoethanol, and 1 U of rth 111 Taq polymerase (Boehringer Mannhiem, CA). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C 1 min, 60°C 1

20   min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

25

Cloning of PSM promoter. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Sysytems, Inc., St. Louis, MI), was screened using a PCR method of Pierce et al. Primers located at the 5' end of PSM

30   cDNA   were   used:5'-CTCAAAAGGGGCCGGATTTCC-3'   and 5'CTCTCAATCTCACTAATGCCTC-3'. A positive clone, p683, was digested with XhoI restriction enzyme. Southern analysis of the restricted fragments using a DNA probe from the extreme 5' to the Ava-1 site of PSM cDNA

35   confirmed   that   a   3Kb   fragment   contains   the   5' regulatory sequence of the PSM gene. The 3 kb XhoI fragment was subcloned into pKSBluescrpt vectors and

sequenced using the dideoxy method.

Functional Assay of PSM Promoter. Chloramphenicol
Acetyl Transferase, (CAT) gene plasmids were
constructed from the Smal-HindIII fragments or
subfragements (using either restriction enzyme
subfragments or PCR) by insertion into promoterless
pCAT basic or pCAT-enhancer vectors (Promega). pCAT-
constructs were cotransfected with pSVßgal plasmid (5
µg of each plasmid) into cell lines in duplicates,
using a calcium phosphate method (Gibco-BRL,
Gaithersburg, MD). The transfected cells were
harvested 72 hours later and assayed (15µg of lysate)
for CAT activity using the LSC method and for ßgal
activity (Promega). CAT activities were standardized
by comparision to that of the ßgal activities.

Results

Sequence of the 5' end of the PSM gene.
The DNA sequence of the 3 kb XhoI fragment of p683
which includes 500 bp of DNA from the RNA start site
was determined (Figures 31A-31D) Sequence 683XFRVS
starts from the 5' distal end of PSM promoter, it
overlaps with the published PSM putative promoter at nt
2485, i.e. the putative transcription start site is at
nt 2485; sequence 683XF107 is the reverse, complement
of 683XFRVS). The sequence from the XhoI fragment
displayed a remarkable arrays of elements and motifs
which are characteristic of eukaryotic promoters and
regulatory regions found in other genes (Figure 32).

**Functional Analysis of upstream PSM genomic elements
for promoter activity.**

Various pCAT-PSM promoter constructs were tested for
promoter activities in two prostatic cell lines:

LNCaP, PC-3 and a colonic SW620 (Figure 33). Induction
of CAT activity was neither observed in p1070-CAT which
contained a 1070 bp PSM 5' promoter fragment, nor in
p676-CAT which contained a 641 bp PSM 5' promoter

5   fragment. However, with an additional SV-40 enhancer,
sequence from -565 to +76 (p676-CATE) exhibited
promoter activity in LNCaP but not in PC-3 nor in
SW620.

10   Therefore, a LNCaP specific promoter fragment from -565
to +76 has been isolated which can be used in PSM
promoter-driven gene therapy.

       EXAMPLE 6:

15

       **ALTERNATIVELY SPLICED VARIANTS OF PROSTATE SPECIFIC
       MEMBRANE ANTIGEN RNA: RATIO OF EXPRESSION AS A
       POTENTIAL MEASUREMENT OF PROGRESSION**

20   MATERIALS AND METHODS

       Cell Lines.   LNCaP and PC-3 prostatic carcinoma cell
       lines were cultured in RPMI and MEM respectively,
       supplemented with 5% fetal calf serum at 37˚C and 5%
25   CO_2.

       Primary tissues.   Primary prostatic tissues were
       obtained from   MSKCC's in-house tumor procurement
       service. Gross specimen were pathologically staged by
30   MSKCC's pathology service.

       RNA Isolation.   Total RNA was   isolated by a
       modified   guanidinium   thiocynate/phenol/chloroform
       method using a RNAzol B kit (Tel-Test, Friendswood,
35   TX). RNA was stored in diethyl pyrocarbonate-treated
       water   at   -80˚C.   RNA   was   quantified   using
       spectrophometric absorption at 260nm.

cDNA synthesis.   Two different batches of normal prostate mRNAs obtained from trauma-dead males (Clontech, Palo Alto, CA) were denatured at 70°C for 10 min., then reverse transcribed into cDNA using random
5   hexamers and Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) at 50°C for 30 min. followed by a 94°C incubation for 5 min.

Polymerase Chain Reaction.   O l i g o n u c l e o t i d e
10   primers (5'-CTCAAAAGGGGCCGGATTTCC-3'   and   5'-AGGCTACTTCACTCAAAG-3'), specific for the 5' and 3' ends of PSM cDNA were designed to span the cDNA sequence. The reaction was performed in a 50 μl volume with a final concentration of the following reagents: 16.6 mM
15   NH$_4$SO$_4$, 67 mM Tris-HCl pH 8.8, acetylated BSA 0.2 mg/ml, 2mM MgCl$_2$, 250μM dNTPs, 10 mM ß-mercaptoethanol, and 1 U of rTth polymerase (Perkin Elmer, Norwalk, CT). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C
20   1 min, 60°C 1 min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

25   **Cloning of PCR products.** PCR products were cloned by the TA cloning method into pCRII vector using a kit from Invitrogen (San Diego, CA). Ligation mixture were transformed into competent *Escherichia coli* Inv5α.

30   **Sequencing.**   Sequencing was   done by the dideoxy method using a sequenase kit from US Biochemical (Cleveland,   OH).   Sequencing   products   were electrophoresed on a 5% polyacrylamide/7M urea gel at 52°C.
35

RNase Protection Assays. Full length PSM cDNA clone was digested with NgoM 1 and Nhe1. A 350 b.p. fragment

was isolated and subcloned into pSPORT1 vector (GIBCO-
BRL, Gaithersburg, MD). The resultant plasmid, pSP350,
was linearized, and the insert was transcribed by SP6
RNA polymerase to yield antisense probe of 395
5 nucleotide long, of which 355 nucleotides and/or 210
nucleotides should be protected from RNAse digestion by
PSM or PSM' RNA respectively (Fig.2). Total celluar
RNA (20 μg) from different tissues were hybridized to
the aforementioned antisense RNA probe. Assays were
10 performed as described (7). tRNA was used as negative
control. RPAs for LNCaP and PC-3 were repeated.

RESULTS

RT-PCR of mRNA from normal prostatic tissue. Two
15 independent RT-PCR of mRNA from normal prostates were
performed as described in *Materials and Methods*.
Subsequent cloning and sequencing of the PCR products
revealed the presence of an alternatively spliced
variant, PSM'. PSM' has a shorter cDNA (2387
20 nucleotides) than PSM (2653 nucleotides). The results
of the sequence analysis are shown in Figure 34. The
cDNAs are identical except for a 266 nucleotide region
near the 5' end of PSM cDNA (nucleotide 114 to 380)
that is absent in PSM' cDNA. Two independent
25 repetitions of RT-PCR of different mRNA samples yielded
identical results.

RNase Protection Assays. An RNA probe complementary to
PSM RNA and spanning the 3' splice junction of PSM' RNA
30 was used to measure relative expression of PSM and PSM'
mRNAs (Figure 35). With this probe, both PSM and PSM'
RNAs in LNCaP cells was detected and the predominant
form was PSM. Neither PSM nor PSM' RNA was detected in
PC-3 cells, in agreement with previous Northern and
35 Western blot data (5,6). Figure 36 showed the
presence of both splice variants in human primary
prostatic tissues. In primary prostatic tumor, PSM is

the dominant form. In contrast, normal prostate expressed more PSM' than PSM. BPH samples showed about equal expression of both variants.

5   **Tumor Index.** The relative expression of PSM and PSM' (Figure 36) was quantified by densitometry and expressed as a tumor index (Figure 37). LNCaP has an index ranging from 9-11; CaP from 3-6; BPH from 0.75 to 1.6; normal prostate has values from 0.075 to 0.45.

10

DISCUSSION
Sequencing data of PCR products derived from human normal prostatic mRNA with 5' and 3' end PSM oligonucleotide primers revealed a second splice

15   variant, PSM', in addition to the previously described PSM cDNA.

PSM is a 750 a.a. protein with a calculated molecular weight of 84,330. PSM was hypothesized to be a type II

20   integral membrane protein (5). A classic type II membrane protein is the transferrin receptor and indeed PSM has a region that has modest homology with the transferrin receptor (5). Analysis of the PSM amino acid sequence by either the methods of Rao and Argos

25   (7) or Eisenburg et. al. (8) strongly predicted one transmembrane helix in the region from a.a.#20 to #43. Both programs found other regions that could be membrane associated but were not considered likely candidates for being transmembrane regions.

30

PSM' antigen, on the other hand, is a 693 a.a. protein as deduced from its mRNA sequence with a molecular weight of 78,000. PSM' antigen lacks the first 57 amino acids present in PSM antigen (Figure 34). It is

35   likely that PSM' antigen is cytosolic.

The function of PSM and PSM' are probably different.

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The cellular location of PSM antigen suggests that it
may interact with either extra- or intra- cellular
ligand(s) or both; while that of PSM' implies that PSM'
can only react with cytosolic ligand(s). Furthermore,
5   PSM antigen has 3 potential phosphorylation sites on
its cytosolic domain. These sites are absent in PSM'
antigen.   On the other hand, PSM' antigen has 25
potential phosphorylation sites, 10 N-myristoylation
sites and 9 N-glycosylation sites. For PSM antigen,
10   all of these potential sites would be on the
extracellular surface. The modifications of these
sites for these homologous proteins would be different
depending on their cellular locations. Consequently,
the function(s) of each form would depend on how they
15   are modified.

The relative differences in expression of PSM and PSM'
by RNase protection assays was analyzed. Results of
expression of PSM and PSM' in primary prostatic tissues
20   strongly suggested a relationship between the relative
expression of these variants and the status of the
cell: either normal or cancerous. While it is noted
here that the sample size of the study is small
(Figures 36 and 37), the consistency of the trend is
25   evident. The samples used were gross specimens from
patients. The results may have been even more dramatic
if specimens that were pure in content of CaP, BPH or
normal had been used.   Nevertheless, in these
specimens, it is clear that there is a relative
30   increase of PSM over PSM' mRNA in the change from
normal to CaP. The Tumor Index (Figure 37) could be
useful in measuring the pathologic state of a given
sample.   It is also possible that the change in
expression of PSM over PSM' may be a reason for tumor
35   progression. A more differentiated tumor state may be
restored by PSM' either by transfection or by the use
of differentiation agents.

References of Example 6

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EXAMPLE 7:

ENHANCED DETECTION OF PROSTATIC HEMATOGENOUS MICRO-
METASTASES WITH PSM PRIMERS AS COMPARED TO PSA PRIMERS
USING A SENSITIVE NESTED REVERSE TRANSCRIPTASE-PCR
ASSAY.

77 randomly selected samples were analyzed from
patients with prostate cancer and reveals that PSM and
PSA primers detected circulating prostate cells in 48
(62.3%) and 7 (9.1%) patients, respectively.   In
treated stage D disease patients, PSM primers detected
cells in 16 of 24 (66.7%), while PSA primers detected
cells in 6 of 24 patients (25%).   In hormone-refractory
prostate cancer (stage D3), 6 of 7 patients were
positive with both PSA and PSM primers.   All six of
these patients died within 2-6 months of their assay,
despite aggressive cytotoxic chemotherapy, in contrast
to the single patient that tested negatively in this
group and is alive 15 months after his assay,
suggesting that PSA-PCR positivity may serve as a
predictor of early mortality.   In post-radical
prostatectomy patients with negative serum PSA values,
PSM primers detected metastases in 21 of 31 patients
(67.7%), while PSA primers detected cells in only 1 of
33 (3.0%), indicating that micrometastatic spread may
be a relatively early event in prostate cancer.   The
analysis of 40 individuals without known prostate
cancer provides evidence that this assay is highly
specific and suggests that PSM expression may predict
the development of cancer in patients without
clinically apparent prostate cancer.   Using PSM
primers, micrometastases were detected in 4 of 40
controls, two of whom had known BPH by prostate biopsy
and were later found to have previously undetected
prostate cancer following repeat prostate biopsy

performed for a rising serum PSA value. These results show the clinical significance of detection of hematogenous micrometastatic prostate cells using PSM primers and potential applications of this molecular 5   assay.

EXAMPLE 8:

MODULATION OF PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) 10 EXPRESSION IN VITRO BY CYTOKINES AND GROWTH FACTORS.

The effectiveness of CYT-356 imaging is enhanced by manipulating expression of PSM. PSM mRNA expression is downregulated by steroids. This is consistent with the 15   clinical observations that PSM is strongly expressed in both anaplastic and hormone refractory lesions. In contrast, PSA expression is decreased following hormone withdrawal. In hormone refractory disease, it is believed that tumor cells may produce both growth 20   factors and receptors, thus establishing an autocrine loop that permits the cells to overcome normal growth constraints.   Many prostate tumor epithelial cells express both TGFα and its receptor, epidermal growth factor receptor. Results indicate that the effects of 25   TGFα and other selected growth factors and cytokines on the expression of PSM in-vitro, in the human prostatic carcinoma cell line LNCaP.

2×10^6 LNCaP cells growing in androgen-depleted media 30   were treated for 24 to 72 hours with EGF, TGFα, TNFß or TNFα in concentrations ranging from 0.1 ng/ml to 100 ng/ml. Total RNA was extracted from the cells and PSM mRNA expression was quantitated by Northern blot analysis and laser densitometry. Both b-FGF and TGFα 35   yielded a dose-dependent 10-fold upregulation of PSM expression, and EGF a 5-fold upregulation, compared to untreated LNCaP. In contrast, other groups have shown

a marked downregulation in PSA expression induced by
these growth factors in this same in-vitro model. TNFα,
which is cytotoxic to LNCaP cells, and TNFß
downregulated PSM expression 8-fold in androgen
5   depleted LNCaP cells.

TGFα is mitogenic for aggressive prostate cancer cells.
There are multiple forms of PSM and only the membrane
form is found in association with tumor progression.
10   The ability to manipulate PSM expression by treatment
with cytokines and growth factors may enhance the
efficacy of Cytogen 356 imaging, and therapeutic
targeting of prostatic metastases.

15   **EXAMPLE 9:**

**NEOADJUVANT ANDROGEN-DEPRIVATION THERAPY (ADT) PRIOR TO
RADICAL PROSTATECTOMY RESULTS IN A SIGNIFICANTLY
DECREASED INCIDENCE OF RESIDUAL MICROMETASTATIC DISEASE
20   AS DETECTED BY NESTED RT-PCT WITH PRIMERS.**

Radical prostatectomy for clinically localized prostate
cancer is considered by many the "gold standard"
treatment. Advances over the past decade have served
25   to decrease morbidity dramatically. Improvements
intended to assist clinicians in better staging
patients preoperatively have been developed, however
the incidence of extra-prostatic spread still exceeds
50%, as reported in numerous studies. A phase III
30   prospective randomized clinical study designed to
compare the effects of ADT for 3 months in patients
undergoing radical prostatectomy with similarly matched
controls receiving surgery alone was conducted. The
previously completed phase II study revealed a 10%
35   margin positive rate in the ADT group (N=69) as
compared to a 33% positive rate (N=72) in the surgery
alone group.

Patients who have completed the phase III study were analyzed to determine if there are any differences between the two groups with respect to residual micrometastatic disease. A positive PCR result in a post-prostatectomy patient identifies viable metastatic cells in the circulation.

Nested RT-PCR was performed with PSM primers on 12 patients from the ADT group and on 10 patients from the control group. Micrometastatic cells were detected in 9/10 patients (90%) in the control group, as compared to only 2/12 (16.7%) in the ADT group. In the ADT group, 1 of 7 patients with organ-confined disease tested positively, as compared to 3 of 3 patients in the control group. In patients with extra-prostatic disease, 1 of 5 were positive in the ADT group, as compared to 6 of 7 in the control group. These results indicate that a significantly higher number of patients may be rendered tumor-free, and potentially "cured" by the use of neoadjuvant ADT.

EXAMPLE 10:

SENSITIVE NESTED RT-PCR DETECTION OF CIRCULATION PROSTATIC TUMOR CELLS - COMPARISON OF PSM AND PSA-BASED ASSAYS

Despite the improved and expanded arsenal of modalities available to clinician today, including sensitive serum PSA assays, CT scan, transrectal ultrasonography, endorectal co.I MRI, etc., many patients are still found to have metastatic disease at the time of pelvic lymph node dissection and radical prostatectomy. A highly sensitive reverse transcription PCR assay capable of detecting occult hematogenous micrometastatic prostatic cells that would otherwise go undetected by presently available staging modalities

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was developed. This assay is a modification of similar
PCR assays performed in patients with prostate cancer
and other malignancies[2,3,4,5]. The assay employs PCR
primers derived from the cDNA sequences of prostate-
specific antigen[6] and the prostate-specific membrane
antigen recently cloned and sequenced.

Materials and Methods

Cells and Reagents.   LNCaP and MCF-7 cells were
obtained from the American Type Culture Collection
(Rockville, MD.). Details regarding the establishment
and characteristics of these cell lines have been
previously published[8,9].   Cells grown in RPMI 1640
medium and supplemented with L-glutamine, nonessential
amino acids, and 5% fetal calf serum (Gibco-BRL,
Gaithersburg, MD.) In a 5% CO_2 incubator at 37°C. All
cell media was obtained from the MSKCC Media
Preparation Facility. Routine chemical reagents were
of the highest grade possible and were obtained from
Sigma Chemical Company (St. Louis, MO).

Patient Blood Specimens. All blood specimens used in
this study were from patients seen in the outpatient
offices of urologists on staff at MSKCC. Two anti-
coagulated tubes per patient were obtained at the time
of their regularly scheduled blood draws. Specimens
were obtained with informed consent of each patient ,
as per a protocol approved by the MSKCC Institutional
Review Board. Samples were promptly brought to the
laboratory for immediate processing. Seventy-seven
specimens from patients with prostate cancer were
randomly selected and delivered to the laboratory
"blinded" along with samples from negative controls for
processing. These included 24 patients with stage D
disease (3 with D_0, 3 with D^1, 11 with D^2, and 7 with
D^3), 31 patients who had previously undergone radical

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prostatectomy and had undetectable postoperative serum
PSA levels (18 with pT2 lesions, 11 with pT3, and 2
pT4), 2 patients with locally recurrent disease
following radical prostatectomy, 4 patients who had
received either external beam radiation therapy or
interstitial l^{125} implants, 10 patients with untreated
clinical stage T1-T2 disease, and 6 patients with
clinical stage T3 disease on anti-androgen therapy.
The forty blood specimens used as negative controls
were from 10 health males, 9 males with biopsy-proven
BPH and elevated serum PSA levels, 7 healthy females,
4 male patients with renal cell carcinoma, 2 patients
with prostatic intraepithelial neoplasia (PIN), 2
patients with transitional cell carcinoma of the
bladder and a pathologically normal prostate, 1 patient
with acute prostatitis, 1 patient with acute
promyelocytic leukemia, 1 patient with testicular
cancer, 1 female patient with renal cell carcinoma, 1
patient with lung cancer, and 1 patient with a cyst of
the testicle.

Blood Sample Processing/RNA Extraction. 4 ml of whole
anticoagulated venous blood was mixed with 3 ml of ice
cold PBS and then carefully layered atop 8 ml of Ficoll
(Pharmacia, Uppsala, Sweden) in a 14-ml polystyrene
tube. Tubes were centrifuged at 200 x g for 30 min. at
4°C. The buffy coat layer (approx. 1 ml.) was
carefully removed and rediluted to 50 ml with ice cold
PBS in a 50 ml polypropylene tube. This tube was then
centrifuged at 2000 x g for 30 min. at 4°C. The
supernatant was carefully decanted and the pellet was
allowed to drip dry. One ml of RNazol B was then added
to the pellet and total RNA was isolated as per
manufacturers directions (Cinna/Biotecx, Houston, TX.)
RNA concentrations and purity were determined by UV
spectroscopy on a Beckman DU 640 spectrophotometer and
by gel analysis.

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Determination of PCR Sensitivity. RNA was isolated from LNCaP cells and from mixtures of LNCaP and MCF-7 cells at fixed ratios (i.e. 1:100, 1:1,000, etc.) using

5   RNAzol B. Nested PCR was then performed as described below with both PSA and PSM primers in order to determine the limit of detection for the assay. LNCaP:MCF-7 (1:100,000) cDNA was diluted with distilled water to obtain concentrations of 1:1,000,000. The

10   human breast cancer cell line MCF-7 was chosen because they had previously been tested by us and shown not to express either PSM nor PSA by both immunohistochemistry and conventional and nested PCR.

15   **Polymerase Chain Reaction.** The PSA outer primer sequences are nucleotides 494-513 (sense) in exon 4 and nucleotides 960-979 (anti-sense) in exon 5 of the PSA cDNA. These primers yield a 486 bp PCR product from PSA CDNA that can be distinguished from a product

20   synthesized from possible contaminating genomic DNA.
PSA-494 5'-TAC CCA CTG CAT CAG GAA CA-3'
PSA-960 5'-CCT TGA AGC ACA CCA TTA CA-3'
The PSA inner upstream primer begins at nucleotide 559 and the downstream primer at nucleotide 894 to yield a

25   355 bp PCR product.
PSA-559 5'-ACA CAG GCC AGG TAT TTC AG-3'
PSA-894 5'-GTC CAG CGT CCA GCA CAC AG-3'
All primers were synthesized by the MSKCC Microchemistry Core Facility. 5μg of total RNA was

30   reverse-transcribed into cDNA using random hexamer primers (Gibco-BRL) and Superscript II reverse transcriptase (Gibco-BRL) according to the manufacturers recommendations. 1μl of this CDNA served as the starting template for the outer primer PCR

35   reaction. The 20μl PCR mix included: 0.5U Taq polymerase (Promega) Promega reaction buffer, 1.5mM MgCl$_2$, 200μM dNTPs, and 1.0μM of each primer. This mix

was then transferred to a Perkin Elmer 9600 DNA thermal cycler and incubated for 25 cycles. The PCR profile was as follows: 94°C x 15 sec., 60°C x 15 sec., and 72°C for 45 sec. After 25 cycles, samples were placed

5   on ice, and 1μl of this reaction mix served as the template for another 25 cycles using the inner primers. The first set of tubes were returned to the thermal cycler for 25 additional cycles. The PSM outer upstream primer sequences are nucleotides 1368-1390 and

10   the downstream primers are nucleotides 1995-2015, yielding a 67 bp PCR product.

PSM-1368 5'-CAG ATA TGT CAT TCT GGG AGG TC-3'
PSM-2015 5'-AAC ACC ATC CCT CCT CGA ACC-3'

15   The PSM inner upstream primer span nucleotides 1689-1713 and the downstream primer span nucleotides 1899-1923, yielding a 234 bp PCR product.
PSM-1689 5'-CCT AAC AAA AGA GCT GAA AAG CCC-3'
PSM-1923 5'-ACT GTG ATA CAG TGG ATA GCC GCT-3'

20   2μl of cDNA was used as the starting DNA template in the PCR assay. The 50μl PCR mix included: 1U Taq polymerase (Boehringer Mannheim), 250μM cNTPs, 10mM ß-mercaptoethanol, 2mM $MgCl_2$, and 5μl of a 10x buffer mix containing: 166mM NH_4SO_4, 670mM Tris pH 8.8, and 2mg/ml

25   of acetylated BSA. PCR was carried out in a Perkin Elmer 480 DNA thermal cycler with the following parameters: 94°C x 4 minutes for 1 cycle, 94°C x 30 sec., 58°C x 1 minute, and 72°C x 1 minute for 25 cycles, followed by 72°C x 10 minutes. Samples were

30   then iced and 2.5μl of this reaction mix was used as the template for another 25 cycles with a new reaction mix containing the inner PSM primers. cDNA quality was verified by performing control reactions using primers derived from the ß-2-microglobulin gene sequence[10] a

35   ubiquitous housekeeping gene. These primers span exons 2-4 and generate a 620 bp PCR product. The sequences for these primers are:

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ß-2 (exon 2) 5'-AGC AGA GAA TGG AAA GTC AAA-3'

ß-2 (exon 4) 5'-TGT TGA TGT TGG ATA AGA GAA-3'

The entire PSA mix and 7-10µl of each PSM reaction mix were run on 1.5-2% agarose gels, stained with ethidium bromide and photographed in an Eage Eye Video Imaging System (Statagene, Torrey Pines, CA.). Assays were repeated at least twice to verify results.

Cloning and Sequencing of PCR Products. PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent E. coli cells using standard methods[11] and plasmid DNA was isolated using Magic Minipreps (Promega) and screened by restriction analysis. Double-stranded TA clones were then sequenced by the dideoxy method[12] using ^{35}S-cCTP (NEN) and Sequenase (U.S. Biochemical). Sequencing products were then analyzed on 6% polyacrilamide/7M urea gels, which were fixed, dried, and autoradiographed as described.

Southern Analysis. PCR products were transferred from ethidium-stained agarose gels to Nytran nylon membranes (Schletcher and Schuell) by pressure blotting with a Posi-blotter (Stratagene) according to the manufacturer's instructions. DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Blots were pre-hybridized at 65°C for 2 hours and subsequently hybridized with denatured ^{32}P-labeled, random-primed[13] cDNA probes (either PSA or PSM). Blots were washed twice in 1x SSC/0.5% SDS at 42°C and twice in 0.1x SSC/0.1% SDS at 50°C for 20 minutes each. Membranes were air-dried and autoradiographed for 1-3 hours at room temperature with Hyperfilm MP (Amersham).

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Results

PSA and PSM N sted PCR Assays: The application of nested PCR increased the level of detection from an average of 1:10,000 using outer primers alone, to better than 1:1,000,000. Dilution curves demonstrating this added sensitivity are shown for PSA and PSM-PCR in Figures 1 and 2 respectively. Figure 1 shows that the 486 bp product of the PSA outer primer set is clearly detectable with ethidium staining to 1:10,000 dilutions, whereas the PSA inner primer 355 bp product is clearly detectable in all dilutions shown. In Figure 2 the PSM outer primer 647 bp product is also clearly detectable in dilutions to only 1:10,000 with conventional PCR, in contrast to the PSM inner nested PCR 234 bp product which is detected in dilutions as low as 1:1,000,000. Southern blotting was performed on all controls and most of the patient samples in order to confirm specificity. Southern blots of the respective dilution curves confirmed the primer specificities but did not reveal any significantly increased sensitivity.

PCR in Negative Controls: Nested PSA and PSM PCR was performed on 40 samples from patients and volunteers as described in the methods and materials section. Figure 48 reveals results from 4 representative negative control specimens, in addition to a positive control. Each specimen in the study was also assayed with the ß-2-microglobulin control, as shown in the figure, in order to verify RNA integrity. Negative results were obtained on 39 of these samples using the PSA primers, however PSM nested PCR yielded 4 positive results. Two of these "false positives" represented patients with elevated serum PSA values and an enlarged prostate who underwent a transrectal prostate biopsy revealing stromal and fibromuscular hyperplasia. In both of

these patients the serum PSA level continued to rise and a repeat prostate biopsy performed at a later date revealed prostate cancer. One patient who presented to the clinic with a testicular cyst was noted to have a
5   positive PSM nested PCR result which has been unable to explain. Unfortunately, this patient never returned for follow up, and thus have not been able to obtain another blood sample to repeat this assay. Positive result were obtained with both PSA and PSM primers in
10   a 61 year old male patient with renal cell carcinoma. This patient has a normal serum PSA level and a normal digital rectal examination. Overall, if the two patients were excluded in whom a positive PCR, but no other clinical test, accurately predicted the presence
15   of prostate cancer, 36/38 (94.7%) of the negative controls were negative with PSM primers, and 39/40 (97.5%) were negative using PSA primers.

Patient Samples: In a "blinded" fashion, in which the
20   laboratory staff were unaware of the nature of each specimen, 117 samples from 77 patients mixed randomly with 40 negative controls were assayed. The patient samples represented a diverse and heterogeneous group as described earlier. Several representative patient
25   samples are displayed in Figure 49, corresponding to positive results from patients with both localized and disseminated disease. Patients 4 and 5, both with stage D prostate cancer exhibit positive results with both the outer and inner primer pairs, indicating a
30   large circulating tumor cell burden, as compared to the other samples. Although the PSM and PSA primers yielded similar sensitivities in LNCaP dilution curves as previously shown, PSM primers detected micrometastases in 62.3% of the patient samples,
35   whereas PSA primers only detected 9.1%. In patients with documented metastatic prostate cancer (stages D_0 - D_3) receiving anti-androgen treatment, PSM primers

detected micrometastases in 16/24 (66.7%), whereas PSA primers detected circulating cells in only 6/24 (25%). In the study 6/7 patients with hormone-refractory prostate cancer (stage D_3) were positive. In the study, PSA primers revealed micrometastatic cells in only 1/15 (6.7%) patients with either pT3 or pT4 (locally-advanced) prostate cancer following radical prostatectomy. PSM primers detected circulating cells in 9/15 (60%) of these patients. Interestingly, circulating cells 13/18 (72.2%) patients with pT2 (organ-confined) prostate cancer following radical prostatectomy using PSM primers was detected. None of these patient samples were positive by PSA-PCR.

Improved and more sensitive method for the detection of minimal, occult micrometastic disease have been reported for a number of malignancies by use of immunohistochemical methods (14), as well as the polymerase chain reaction (3, 4, 5). The application of PCR to detect occult hematogenous micrometastases in prostate cancer was first described by Moreno, et al. (2) using conventional PCR with PSA-derived primers.

When human prostate tumors and prostate cancer cells in-vitro were studied by immunohistochemistry and mRNA analysis, PSM appeared to be highly expressed in anaplastic cells, hormone-refractory cells, and bony metastases (22, 23, 24), in contrast to PSA. If cells capable of hematogenous micrometastasis represent the more aggressive and poorly-differentiated cells, they may express a higher level of PSM per cell as compared to PSA, enhancing their detectibility by RT-PCR.

Nested RT-PCR assays are both sensitive and specific. Results have been reliably reproduced on repeated occasions. Long term testing of both cDNA and RNA stability is presently underway. Both assays are

capable of detecting one prostatic cell in at least one million non-prostatic cells of similar size. This confirms the validity of the comparison of PSM vs. PSA primers. Similar levels of PSM expression in both

5   human prostatic cancer cells in-vivo and LNCaP cells in-vitro resulted. The specificity of the PSM-PCR assay was supported by the finding that two "negative control" patients with positive PSM-PCR results were both subsequently found to have prostate cancer. This

10   suggests an exciting potential application for this technique for use in cancer screening. In contrast to recently published data (18), significant ability for PSA primers to accurately detect micrometastatic cells in patients with pathologically with pathologically

15   organ-confined prostate cancer, despite the sensitivity of the assay failed to result. Rather a surprisingly high percentage of patients with localized prostate cancer that harbor occult circulating prostate cells following "curative" radical prostatectomy results

20   which suggests that micrometastasis is an early event in prostate cancer.

     The application of this powerful new modality to potentially stage and/or follow the response to therapy

25   in patients with prostate cancer certainly merits further investigation. In comparison to molecular detection of occult tumor cells, present clinical modalities for the detection of prostate cancer spread appear inadequate.

30

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EXAMPLE 11:

CHROMOSOMAL LOCALIZATION OF COSMID CLONES 194 AND 683 BY FLUORESCENCE IN-SITU HYBRIDIZATION:

PSM was initially mapped as being located on chromosome 11p11.2-p13 (Figures 51-54). Further information from the cDNA in-situ hybridizations experiments demonstrated as much hybridization on the q as p arms. Much larger fragments of genomic DNA was obtained as cosmids and two of these of about 60 kilobases each one going 3' and the other 5' both demonstrated binding to chromosome 11 p and q under low stringency. However under higher stringency conditions only the binding at 11q14-q21 remained. This result suggests that there is another gene on 11p that is very similar to PSM because it is so strongly binding to nearly 120 kilobases of genomic DNA (Figure 50).

Purified DNA from cosmid clones 194 and 683 was labelled with biotin dUTP by nick translation. Labelled probes were combined with sheared human DNA and independently hybridized to normal metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dectran sulfate, and 2XSSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresein conjugated avidin. Following signal detection the slides were counterstained with propidium iodide and analyzed. These first experiments resulted in the specific labelling of a group C chromosome on both the long and short arms. This chromosome was believed to be chromosome 11 on the basis of its size and morphology. A second set of experiments were performed in which a chromosome 11 centromere specific probe was

cohybridized with the cosmid clones. These experiments were carried out in 60% formamide in an attempt to eliminate the cross reactive signal which was observed when low stringency hybridizations were done. These experiments resulted in the specific labelling of the centromere and the long arm of chromosome 11. Measurements of 10 specifically labelled chromosomes 11 demonstrated that the cosmid clones are located at a position which is 44% of the distance from the centromere to the telomere of chromosome arm 11q, an area that corresponds to band 14q. A total of 160 metaphase cells were examined with 153 cells exhibiting specific labelling.

Cloning of the 5' upstream and 3' downstream regions of the PSM genomic DNA. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Systems, St. Louis, MI) was screened using the PCR method of Pierce et. al. Primer pairs located at either the 5' or 3' termini of PSM cDNA were used. Positive cosmid clones were digested with restriction enzymes and confirmed by Southern analysis using probes which were constructed from either the 5' or 3' ends of PSM cDNA. Positive clone p683 contains the 5' region of PSM cDNA and about 60 kb upstream region. Clone -194 contains the 3' terminal of the PSM cDNA and about 60 kb downstream.

EXAMPLE 12:

PEPTIDASE ENZYMATIC ACTIVITY

PSM is a type two membrane protein. Most type two membrane proteins are binding proteins, transport proteins or peptidases. PSM appears to have peptidase activity. When examining LNCaP cells with a substrate N-acetyl-aspartyl-^{14}C-glutamic acid, NAAG, glutamic acid was released, thus acting as a carboxypeptidase. In

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vitro translated PSM message also had this peptidase
activity..

The result is that seminal plasma is rich in its
5   content of glutamic acid, and are able to design
inhibitors to enhance the activity of the non degraded
normal substrate if its increased level will have a
biologic desired activity. Also biologic activity can
be measured to see how it correlates wit the level of
10   message. Tissue may be examined for activity directly
rather than indirectly using in-situ analysis or
immunohistochemical probes. Because there is another
gene highly similar on the other arm of chromosome 11
when isolated the expressed cloned genes can be used to
15   determine what are the substrate differences and use
those substrates for identification of PSM related
activity, say in circulating cells when looking for
metastases.

20   **EXAMPLE 13:**

**IONOTROPICGLUTAMATE RECEPTOR DISTRIBUTION IN PROSTATE
TISSUE**

25   **Introduction:**
Excitatory neurotransmission in the central nervous
system (CNS) is mediated predominantly by glutamate
receptors. Two types of glutamate receptors have been
identified in human CNS: metabotropic receptors, which
30   are coupled to second-messenger systems, and ionotropic
receptors, which serve as ligand-gated ion channels.
The presence of ionotropic glutamate receptors in human
prostate tissue was investigated.

35   **Methods:**
Detection of glutamate receptor expression was
performed using anti-GluR2/3 and anti-biotin

immunohistochemical technique in paraffin-embedded
human prostate tissues. PSM antigen is a
neurocarboxypeptidase that acts to release glutamate.
In the CNS glutamate acts as a neurotransmitter by
acting on glutaminergic ion channels and increases the
flow of ions like calcium ions. One way the glutamate
signal is transduced into cell activity is the
activation of nitric oxide synthase, and nitric oxide
synthase has recently been found to be present in human
prostatic tissue. NO is a major signalling mechanism
and is involved in control of cell growth and death, in
response to inflammation, in smooth muscle cell
contraction, etc,. In the prostate much of the stroma
is smooth muscle. It was discovered that the prostate
is rich in glutaminergic receptors and have begun to
define this relationship. Stromal abnormalities are
the key feature of BPH. Stromal epithelial
interactions are of importance in bothe BPH and CaP.
The other glutaminergic receptors through G proteins to
change the metabolism of the cell.

Results:
Anti-GluR2/3 immunoreactivity was unique to prostatic
stroma and was absent in the prostatic epithelial
compartment. Strong anti-GluR4 immunoreactivity was
observed in basal cells of prostatic acini.

Discussion:
The differential distribution of ionotropic glutamate
receptor subtypes between the stromal and epithelial
compartments of the prostate has not been previously
described. Prostate-specific membrane antigen
(PSMA) has an analogous prostatic distribution, with
expression restricted to the epithelial compartment.

PSM antigen is a neurocarboxypeptidase that acts to

release glutamate from NAAG 1, also a potential
nerotransmitter.   In the CNS glutamate acts as a
neurotransmitter by acting on glutaminergic ion
channels and increases the flow of ions like calcium
5   ions. One way the glutamate signal is transduced into
cell activity is the activation of nitric oxide
synthase, and nitric oxide synthase has recently been
found to be present in human prostatic tissue. NO is
a major signaling mechanism and is involved in control
10   of cell growth and death, in response to inflammation,
in smooth muscle cell contraction, etc,.   In the
prostate much of the stroma is smooth muscle.   The
prostate is rich in glutaminergic receptors. Stromal
abnormalities are the key feature of BPH.   Stromal
15   epithelial interactions are of importance in both BPH
and CaP. The other glutaminergic receptors through G
proteins to change the metabolism of the cell.
Glutamate can be produced in the cerebral cortex
through the carboxypeptidase activity of the prostate-
20   specific membrane antigen (PSMA).   In this location,
PSMA cleaves glutamate from acetyl-aspartyl-glutamate.
Taken together, these observations suggest a function
for PSMA in the human prostate; glutamate may be an
autocrine and/or paracrine signalling molecule,
25   possibly mediating epithelial-stromal interactions.
Ionotropic glutamate receptors display a unique
compartmental distribution in the human prostate.

The carboxypeptidase like activity and one substrate is
30   the dipeptide N-acetyl-aspartyl glutamic acid, NAAG
which is one of the best substrates found to date to
act as a neurotransmitter in the central nervous system
and its abnormal function may be associated with
neurotoxic disorder such as epilepsy, ALS, alzheimers
35   etc.   PSM carboxypeptidase may serve to process
neuropeptide transmitters in the prostate.
Neuropeptide transmitters are associated with the

neuroendocrine cells of the prostate and neuroendocrine cells and are thought to play a role in prostatic tumor progression. Interestingly PSM antigen's expression is upregulated in cancer. Peptides known to act as prostatic growth factors such as TGF-a and bFGF, up regulate the expression of the antigen. TNF on the other hand downregulate PSM. TGF and FGF act through the mitogen activated signaling pathway, while TNF acts through the stress activated protein kinase pathway. Thus modulation of PSM expression is useful for enhancing therapy.

EXAMPLE 14:

IDENTIFICATION OF A MEMBRANE-BOUND PTEROYLPOLYGAMMA-GLUTAMYL CARBOXYPEPTIDASE (FOLATE HYDROLASE) THAT IS EXPRESSED IN HUMAN PROSTATIC CARCINOMA

PSM may have activities both as a folate hydrolase and a carboxyneuropeptidase. For the cytotoxic drug methotrexate to be a tumor toxin it has to get into the cell and be polygammaglutamated which to be active, because polyglutamated forms serve as the enzyme substrates and because polyglutamated forms or toxins are also retained by the cell. Folate hydrolase is a competing reaction and deglutamates methotrexate which then can diffuse back out of the cell. Cells that overexpose folate hydrolase activity are resistant to methotrexate. Prostate cancer has always been absolutely refractory to methotrexate therapy and this may explain why, since the prostate and prostate cancer has a lot of folate hydolase activity. However, based on this activity, prodrugs may be generated which would be activate at the site of the tumor such as N-phosphonoacetyl-1-aspartate-glutamate. PALglu is an inhibitor of the enzyme activity with NAAG as a substrate.

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Prostate specific membrane antigen was immuno precipitated from the prostate cancer cell line LNCaP and demonstrated it to be rich in folate hydolase activity, with gammaglutamated folate or polyglutamated

5   methotrexate being much more potent inhibitors of the neuropeptidase activity than was quisqualate, which was the most potent inhibitor reported up to this time and consistent with the notion that polyglutamated folates may be the preferred substrate.

10

Penta-gammaglutamyl-folate is a very potent inhibitor of activity (inhibition of the activity of the enzyme is with 0.5um Ki.) As penta-gammaglutamyl-folate may also be a substrate and as folates have to be

15   depolygammaglutamated in order to be transported into the cell, this suggest that this enzyme may also play a role in folate metabolism. Folate is necessary for the support of cell function and growth and thus this enzyme may serve to modulate folate access to the

20   prostate and prostate tumor. The other area where PSM is expressed is in the small intestine. It turns out that a key enzyme of the small intestine that is involved in folate uptake acts as a gamma-carboxypeptidase in sequentially proteolytically

25   removing the terminal gammaglutaminyl group from folate. In the bone there is a high level of unusual gammaglutamate modified proteins in which the gamma glutamyl group is further carboxylated to produce gammacarboxyglutamate, or GLA. One such protein is

30   osteonectin.

Using capillary electrophoresisis pteroyl poly-gamma-glutamate carboxypeptidase (hydrolase) activity was investigated in membrane preparations from androgen-

35   sensitive human prostatic carcinoma cells (LNCaP). The enzyme immunologically cross-reacts with a derivative of an anti-prostate monoclonal antibody (7E11-C5) that

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recognizes prostate specific membrane (PSM) antigen.
The PSM enzyme hydrolyzes gamma-glutamyl linkages and
is an exopeptidase as it liberates progressively
glutamates from methotrexate triglutamate (MTXGlu$_3$)

5   and folate pentaglutamate (Pte Glu$_5$) with accumulation
of MTX and Pte Glu respectively. The semi-purified
membrane-bound enzyme has a broad activity from pH 2 to
10 and is maximally active at pH4.0. Enzymatic activity
was weakly inhibited by dithfothreitol ($\geq$0.2 mM) but

10   not by reduced glutathione, homocysteine, or p-
hydroxymercuribenzoate (0.05-0.5 mM). By contrast to
LNCaP cell membranes, membranes isolated from androgen-
insensitive human prostate (TSU-Prl, Duke-145, PC-3)
and estrogen-sensitive mammary adenocarcinoma (MCF-7)

15   cells do not exhibit comparable hydrolase activity nor
do they react with 7E11-C5. Thus, a folate hydrolase
was   identified in LNCap cells   that   exhibits
exopeptidase activity and is strongly expressed by
these cells.

20

PALA-Glutamate 3 was tested for efficacy of the prodrug
strategy by preparing N-acetylaspartylglutamate, NAAG
1(Figure 59). NAAG was synthesized from commercially
available gamma-benzylaspartate which was acetylated

25   with acetic anhydride in pyridine to afford N-acetyl-
gamma-benzyl aspartate in nearly quantitative yield.
The latter was activated as its pentafluorophenyl ester
by treatment with pentafluorophenyltrifluoroacetate in
pyridine at 0 deg.C for an hour. This activated ester

30   constitutes the central piece in the preparation of
compounds 1 and 4 (Figure 60). When 6 is reacted with
epsilon-benzyl-L-glutamate in the presence of HOAT(1-
hydroxy-7-azabenzotriazole)   in   THF-DMF
(tetrahydrofuran, N,N- dimethylformamide) at reflux for

35   an overnight period and after removal of the benzyl
protecting groups by hydrogenolysis (H2, 30 psi, 10%
Pd/C in ethylacetate) gave a product which was

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identical in all respects to commercially available
NAAG (Sigma).

PALA-Glutamate 3 and analog 5, was synthesized in a
similar manner with the addition to the introduction of
a protected phosphonoacetate moiety instead of a simple
acetate.   It is compatible with the function   of
diethylphosphonoacetic acid which allows the removal of
the ethyl groups under relatively mild conditions.

Commercially available diethylphosphonoacetic acid was
treated with perfluorophenyl acetate in pyridine at 0
deg.C to room temperature for an hour to afford the
corresponding pentafluorophenyl ester in nearly
quantitative yield after short path column
chromatography. This was then reacted with gamma-
benzylaspartate and HOAT in tetrahydrofuran for half an
hour at reflux temperature to give protected PALA 7 (N-
phosphonoacetylaspartate) in 90% yield after flash
column chromatography.   The free acid was then
activated as its pentafluorophenyl ester 8, then it was
reacted with delta-benzyl-L-glutamate and HOAT in a
mixture of THF-DMF (9:1, v/v) for 12 hours at reflux to
give fully protected PALA-Glutamate 9 in 66% yield
after column chromatography. Sequential removal of the
ethyl groups followed by the debenzylation was
accomplished for a one step deprotection of both the
benzyl and ethyl groups.   Hence protected PALA-
Glutamate was heated up to reflux in neat
trimethylsilylchloride for an overnight period. The
resulting bistrimethylsilylphosphonate ester 10 was
submitted without purification to hydrogenolysis (H_2 30
psi, 10% Pd/C, ethylacetate). The desired material 3
was isolated after purification by reverse phase column
chromatography and ion exchange resin.

Analogs 4 and 5 were synthesized by preparation of

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phosphonoglutamate 14 from the alpha-carboxyl-protected glutamate.

Commercially available alpha-benzyl-N-Boc-L-glutamate 11 was treated at refluxing THF with neat boranedimethylsulfide complex to afford the corresponding alcohol in 90% yield. This was transformed into bromide 12 by the usual procedure (Pph_3, CBr_4).

The Michaelis-Arbuzov reaction using triethylphosphite to give the corresponding diethylphosphonate 13 which would be deprotected at the nitrogen with trifluoroacetic acid to give free amine 14. The latter would be condensed separately with either pentafluorophenylesters 6 or 8 to give 16 and 15 respectively, under conditions similar to those described for 3. 15 and 16 would be deprotected in the same manner as for 3 to yield desired analogs 4 and 5.

An inhibitor of the metabolism of purines and pyrimidine like DON (6-diazo-5-oxo-norleucine) or its aspartate-like 17, and glutamate-like 18 analogs would be added to the series of substrates.

Analog 20 is transformed into compound 17 by treatment with oxalyl chloride followed by diazomethane and deprotection under known conditions to afford the desired analogs. In addition, azotomycin is active only after in vivo conversion to DON which will be released after action of PSM on analogs 17, 18, and 19.

In addition, most if not all chemotherapies rely on one hypothesis; fast growing cells possess a far higher appetite for nutrients than normal cells. Hence, they uptake most of the chemotherapeutic drugs in their proximity. This is why chemotherapy is associated with

serious secondary effects (weakening of the immune
system, loss of hair, ...) that sometimes put the
patient's life in danger. A selective and effective
drug that cures where it should without damaging what
5   it shouldn't damage is embodied in representative
structures 21 and 22.

Representative compounds, 21 and 22, were designed
based on some of the specific effects and properties of
10   PSM, and the unique features of some newly discovered
cytotoxic molecules with now known mode of action. The
latter, referred to commonly as enediynes, like
dynemycin A 23 and or its active analogs. The recent
isolation of new natural products like Dynemycin A 23,
15   has generated a tremendous and rapidly growing interest
in the medical and chemical sciences. They have
displayed cytotoxicities to many cancer cell lines at
the sub-nanomolar level. One problem is they are very
toxic, unstable, and non-selective. Although they have
20   been demonstrated, in vitro, to exert their activity
through DNA damage by a radical mechanism as described
below, their high level of toxicity might imply that
they should be able to equally damage anything in their
path, from proteins to enzymes, ...etc.
25

These molecules possess unusual structural features
that provide them with exceptional reactivities.
Dynemycin A 23 is relatively stable until the
anthraquinone moiety is bioreduced into
30   hydroanthraquinone 24. This triggers a chain of events
by which a diradical species 25 is generated as a
result of a Bergman cycloaromatization[f]. Diradical
species 25 is the ultimate damaging edge of dynemycin
A. It subtracts 2(two) protons from any neighboring
35   molecule or molecules(ie. DNA) producing radicals
therein. These radicals in turn combine with molecular
oxygen to give hydroperoxide intermediates that, in the

-142-

case of DNA, lead to single and double strand incision, and consequent cell death. Another interesting feature was provided by the extensive work of many organic chemists who not only achieved the total synthesis of (+)-dynemycin A 23 and other enediynes. but also designed and efficiently prepared simpler yet as active analogs like 26.

Enediyne 26 is also triggerable and acts by virtue of the same mechanism as for 23. This aspect is very relevant to the present proposed study in that 27 (a very close analog of 26) is connected to NAAG such that the NAAG-27 molecule, 21, would be inert anywhere in the body (blood, organs, normal prostate cells, ...etc.) except in the vicinity of prostate cancer, and metastatic cells. In this connection NAAG plays a multiple role:

- Solubilization and transport: analogs of 26-type are hydrophobic and insoluble in aqueous media, but with a water soluble dipeptide that is indigenous to the body, substrate 21 should follow the ways by which NAAG is transported and stored in the body.

- Recognition, guidance, and selectivity: Homologs of PSM are located in the small intestines and in the brain.

In the latter, a compound like 27 when attached to a multiply charged dipeptide like NAAG, has no chance of crossing the blood brain barrier. In the former case, PSM homolog concentration in the small intestines is very low compared to that of PSM in prostrate cancer cells. In addition, one could enhance the selectivity of delivery of the prodrug by local injection in the prostate. Another image of this strategy could be formulated as follows. If prostate cancer were a war

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in which one needed a "smart bomb" to minimize the
damage within the peaceful surroundings of the war
zone, then 21 would be that "smart bomb". NAAG would
be its guidance system, PSM would be the trigger, and
5   27 would be the warhead.

26 and its analogs are established active molecules
that portray the activity of dynemycin A. Their
syntheses are described in the literature. The total
10   synthesis of optically active 27 has been described[G].
The synthetic scheme that for the preparation of 28 is
almost the same as that of 27. However, they differ
only at the position of the methoxy group which is meta
to the nitrogen in the case of 28. This requires an
15   intermediate of type 29, and this is going to be
prepared by modification of the Myers' method.
Compound 28 is perhaps the closest optically active
analog that resembles very much 26, and since the
activity of the latter is known and very high.
20

Since NAAG is optically pure, its combination with
racemic material sometimes complicates purification of
intermediates. In addition, to be able to modify the
components of this system one at a time, optically pure
25   intermediates of the type 21 and 22 are prepared. 27
was prepared in 17 steps starting fro commercially
available material. Another interesting feature of 27
is as demonstrates in a very close analog 26, it
possesses two(2) triggers as shown by the arrows.
30

The oxygen and the nitrogen can both engender the
Bergman cycloaromatization and hence the desired
damage. The simple protection deprotection
manipulation of either functionality should permit the
35   selective positioning of NAAG at the nitrogen or at the
oxygen centers. PSM should recognize the NAAG portion
of 21 or 22, then it would remove the glutamic acid

moiety.   This leaves 27 attached to N-acetylaspartate.

Intramolecular assisted hydrolysis of systems like N-
acetylaspartyle is well documented in the literature.
5   The aminoacid portion should facilitate the hydrolysis
of such a linkage.   In the event this would not work
when NAAG is placed on the nitrogen, an alternative
would be to attach NAAG to the oxygen giving rise to
phenolic ester 22 which is per se labile and removable
10   under milder conditions.   PSM specific substrates can
be designed that could activate pro-drugs at the site
of prostatic tumor cells to kill those cells. PSM
specific substrates may be used in treatment of benign
prostatic hyperplasia.

EXAMPLE 15:

GENOMIC ORGANIZATION OF PSM EXON/INTRON JUNCTION
5   **SEQUENCES**

                    EXON 1   Intron 1
        1F. strand
        CGGCTTCCTCTTCGG
10   cggcttcctcttcgg   tagggggggcgcctcgcggag...tatttttca

        1R. strand   ...ataaaaagtCCCACCAAA

15   Exon 2   Intron 2
        2F. strand
        ACATCAAGAAGTTCT
        acatcaagaagttct   caagtaagtccatactcgaag...

20   2R. strand   ...caagtggtcATTAAAATG

                    Exon 3   Intron 3
        3F. strand
25   GAAGATGGAAATGAG
        gaagatggaaatgag   gtaaaatataaataaataaataa...

                    Exon 4   Intron 4
30   4F. strand
        AAGGAATGCCAGAGG
        aaggaatgccagagg   taaaaacacagtgcaacaaa...

        4R. strand   ...agagttgTCCCGCTAGAT
35

                    Exon 5   Intron 5
         5F. strand
         CAGAGGAAATAAGGT
         cagaggaaataaggt aggtaaaaattatctctttttt...
  5   ...gtgttttctAGGTTAAAAATG

         5R. strand   ...cacttttgaTCCAATTT

  10   Exon 6   Intron 6
         6F. strand
         GTTACCCAGCAAATG
         gttacccagcaatg   gtgaatgatcaatccttgaat...

  15   6R. strand   ...aaaaaaagtCTTATACGAATA

                    Exon 7   Intron 7
         7F. strand
  20   ACAGAAGCTCCTAGA
         acagaagctcctaga   gtaagtttgtaagaaaccargg...

         7R. strand   ...aaacacaggttatcTTTTTACCCA

  25   Exon 8   Intron 8
         8F. strand
         AAACTTTTCTACACA
         aaacttttctacaca   gttaagagactatataaattttta...

  30   8R. strand   ....aaacgtaatcaTTTTCAGTTCTAC

                    Exon 9   Intron 9
         9F. strand
         AGCAGTGGAACCAG
  35   agcagtggaaccag gtaaaggaatcgtttgctagca...
                                    ...tttctagatAGATATGTCATTC

```
9R. strand                              ...aaagaTCTGTCTATACAGTAA


            Exon 10           Intron 10
      10F. Strand
  5   CTGAAAAAGGAAGG
      ctgaaaaaggaagg      taatacaaacaaatagcaagaa...



            Exon 11           Intron 11
 10   11F. Strand
      TGAGTGGGCAGAGG
               agagg      ttagttggtaatttgctataatata...



 15         Exon 13           Intron 12
      12R. strand
      GAGTGTAGTTTCCT
           gtagtttcct      gaaaaataagaaaagaatagat...


 20
            Exon 14           Intron 13
      13R. strand
      AGGGCTTTTCAGCT
      agggcttttcagct      acacaaattaaaagaaaaaaag...
 25


            Exon 14           Intron 14
      14F. strand
      GTGGCATGCCCAGG
 30   gtggcatgcccagg      taaataaatgaatgaagtttcca...


            Exon 16           Intron 15
      15R. strand
      AATTTGTTTGTTTCC
 35   aatttgtttgtttcc      tacagaaaaaacaacaaaaca...
```

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```
              Exon 16          Intron 16
      16F. strand
      CAGTGTATCATTTG
      cagtgtatcatttg  gtatgttccccttcctttttcaaatt...
  5                                 ...tttcagATTCACTTTTTT


      16R. strand                   ...aaagtcTAAGTGAAAA



 10            Exon 17          Intron 17
      17F. strand
      TTTGACAAAAGCAA
      tttgacaaaagcaa      gtatgttctacatatatgtgcatat...

 15   17R. strand                    ...aaagagtcGGGTTA




              Exon 18          Intron 18
      18F. strand
 20   GGCCTTTTTATAGG
      ggccttttatagg      taaganaagaaaatatgactcct...


      18R. strand                      ...aatagttgTGTAAACCC


 25
              Exon 19          Intron 19
      19F. strand
      GAATATTATATATA
      gaatattatatata      gttatgtgagtgtttatatatgtgtgt...
 30


      Notes: F: Forward strand
             R: Reverse strand



 35
```

What is claimed is:

1.   An isolated nucleic acid molecule encoding an alternatively spliced prostate-specific membrane (PSM') antigen.

2.   An isolated mammalian DNA molecule of claim 1.

3.   An isolated mammalian cDNA molecule of claim 2.

4.   An isolated mammalian RNA molecule derived from claim 1.

5.   An isolated nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the isolated nucleic acid molecule of claim 1.

6.   A DNA molecule of claim 5.

7.   A RNA molecule of claim 5.

8.   A method of detecting expression of a alternatively spliced prostate-specific membrane (PSM') antigen in a cell which comprises obtaining total mRNA from the cell, contacting the mRNA so obtained with a labelled nucleic acid molecule of claim 5 under hybridizing conditions, determining the presence of mRNA hybridized to the molecule, and thereby detecting the expression of the alternatively spliced prostate-specific membrane (PSM') antigen in the cell.

9.   An isolated nucleic acid molecule of claim 2 operatively linked to a promoter of RNA transcription.

10.   A vector which comprises the isolated nucleic
        acid molecule of claim 1.

11.   A host vector system for the production of a
        polypeptide having the biological activity of the
        alternatively spliced prostate-specific membrane
        (PSM') antigen which comprises the vector of
        claim 10 and a suitable host.

12.   A host vector system of claim 11, wherein the
        suitable host is a bacterial cell, insect cell,
        or mammalian cell.

13.   A method of producing a polypeptide having the
        biological activity of the prostate-specific
        membrane antigen which comprises growing the host
        cells of the host vector system of claim 12 under
        suitable conditions permitting production of the
        polypeptide and recovering the polypeptide so
        produced.

14.   An isolated nucleic acid molecule encoding a
        prostate-specific membrane antigen promoter.

15.   A polypeptide encoded by the isolated nucleic
        acid molecule of claim 1.

16.   A method of detecting hematogenous micrometastic
        tumor cells of a subject, comprising (A)
        performing nested polymerase chain reaction (PCR)
        on blood, bone marrow or lymph node samples of
        the subject using the prostate specific membrane
        antigen primers, and (B) verifying
        micrometastases by DNA sequencing and Southern
        analysis, thereby detecting hematogenous
        micrometastic tumor cells of the subject.

17. The method of claim 16, wherein the primers are derived from prostate specific antigen.

18. The method of claim 16, wherein the subjects is administered hormones, epidermal growth factor, b-fibroblast growth factors, or tumor necrosis factor.

19. A method of determining prostate cancer progression in a subject which comprises: a) obtaining a suitable prostate tissue sample; b) extracting RNA from the prostate tissue sample; c) performing a RNAse protection assay on the RNA, thereby forming a duplex RNA-RNA hybrid; d) detecting PSM and PSM' amounts in the tissue sample; e) calculating a PSM/PSM' tumor index, thereby determining prostate cancer progression in the subject.

20. The method of claim 19, further comprising performing in-situ hyribridization.
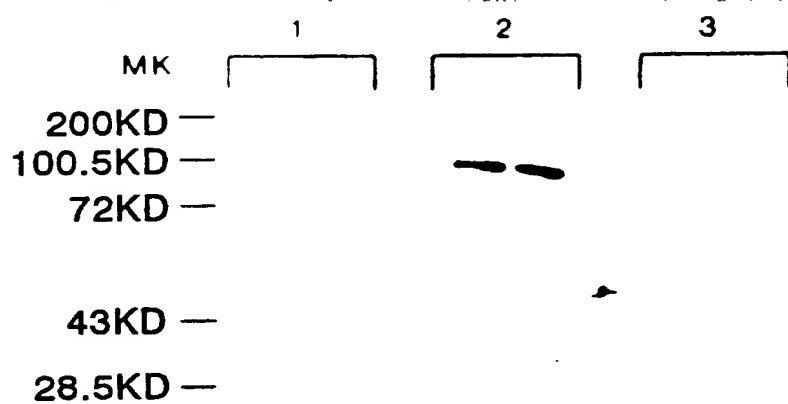
FIGURE 1


```
1 - anti- EGFr PoAB RK-2
2 - Cyt-356 MoAB/RAM
3 - RAM
```

FIGURE 2A

FIGURE 2B

FIGURE 2C

FIGURE 2D

FIGURE 3B

FIGURE 3D

FIGURE 3A

FIGURE 3C

FIGURE 4

100.5 ▬▬   ▬▬▬

72.0 ▬▬▬

43.0 ▬▬▬

28.5 ▬▬▬

FIGURE 5

FIGURE 6A

FIGURE 6B

FIGURE 7

FIGURE 8

FIGURE 9

```
  4 —
  3 —
  2 —
1.6 —
```

FIGURE 10

FIGURE 11

FIGURE 12A FIGURE 12B

FIGURE 13

FIGURE 14-1

Done on sequence PMSANTIGEN.
Total number of residues is: 750.
Analysis done on the complete sequence.

```
In Helical   (H)  conformation  [DC =  -75  CNAT ]  :  264 AA  =>  35.2%
In Extended  (E)  conformation  [DC =  -88  CNAT ]  :  309 AA  =>  41.2%
In  Turn     (T)  conformation  [DC =    0  CNAT ]  :   76 AA  =>  10.1%
In  Coil     (C)  conformation  [DC =    0  CNAT ]  :  101 AA  =>  13.4%
```

Sequence shown with conformation codes.
==

Consecutive stretch of 5 or more residues in a given conformation are
overlined.

```
 1  HHHHHHHHHHHHHHHHHHHHEETTEEEEEEEE

31  EEEEHHHHHHHHHHCCCCCTHHHHHHHHHHHH

61  HHHHHHHHHHHHHEEETTEEEEECCCCCHHHHH
```

FIGURE 14-2

91
121
151
181
211
241
271
301
331
361
391
421

FIGURE 14-3

451
481
511
541
571
601
631
661
691
721
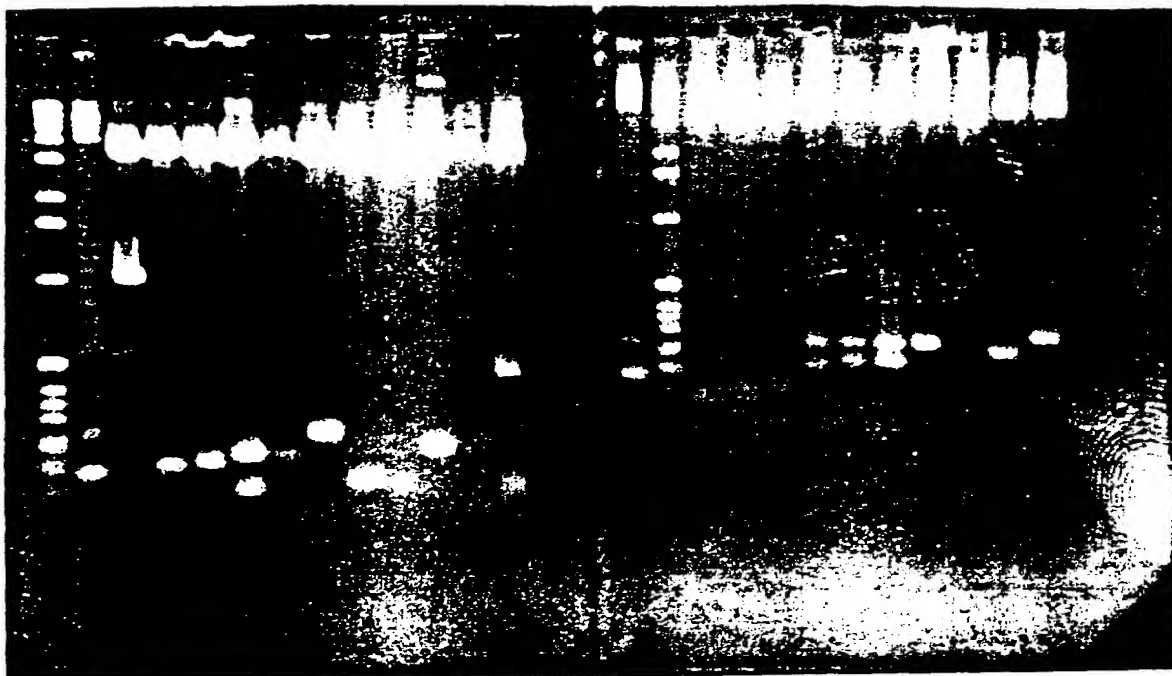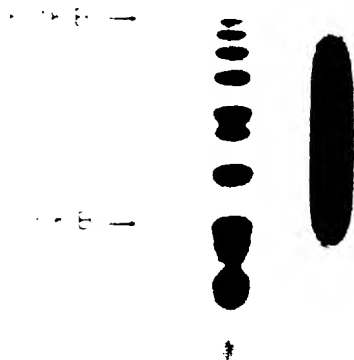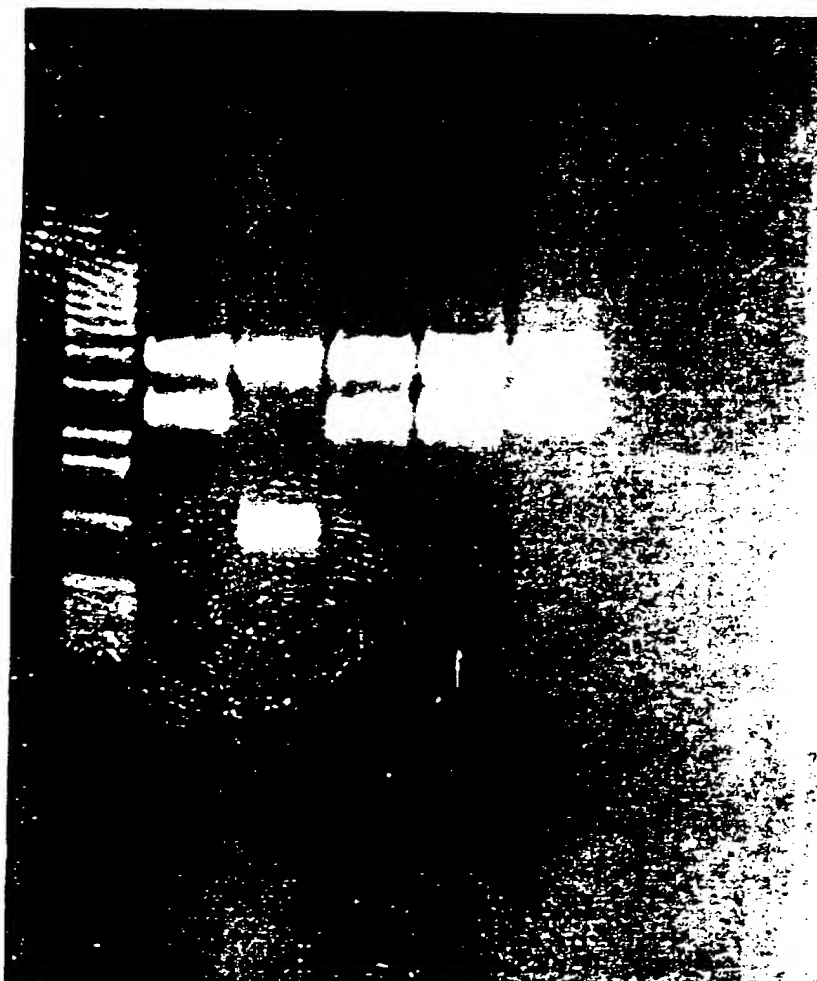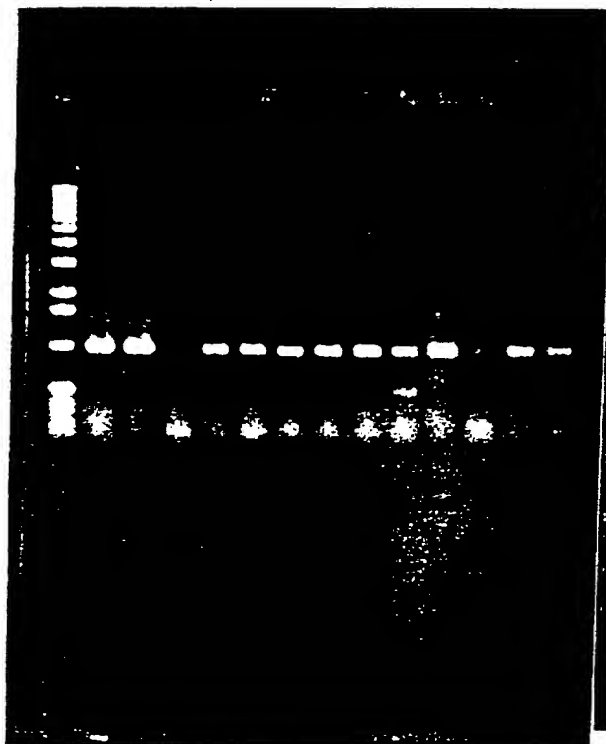
FIGURE 14-4

Semi-graphical output.
===========================

Symbols used in the semi-graphical representation:

Helical conformation: X   Extended conformation: -
Turn conformation: >   Coil conformation: *

```
           10        20        30        40        50
            -         -         -         -         -
MWNLLHETDSAVATARRPRWLCAGALVLAGGFFLLGFLFGWFIKSSNEAT

XXXXXXXXXXXXXXX---->>-------------------XXXXXX*****>X
XXXXXXXXXXXXXXX---->>-------------------XXXXXX******>X

           60        70        80        90        100
            -         -         -         -         -
NITPKHNMKAFLDELKAENIKKFLYNFTQIPHLAGTEQNFQLAKQIQSQW
```

FIGURE 14-5

```
XXXXXXXXXXXXXXXXX---->>---***XXXXXXXXX-X*--
XXXXXXXXXXXXXXX------>>---***XXXXXXXX--X*--
    110      120      130      140      150
KEFGLDSVELAHYDVLLSYPNKTHPNYISIINEDGNEIFNTSLFEPPPPG

-->>**XXXXXXXX------>>**----*>**X---->>***>>
-->>**XXXXXXXX------>>**----*>**X---->>***>>
    160      170      180      190      200
YENVSDIVPPFSAFSPQGMPEGDLVYVNYARTEDFFKLERDMKINCSGKI
```

FIGURE 14-6

VIARYGKVFRGNKVKNAQLAGAKGVILYSDPADYFAPGVKSYPDGWNLPG

GGVQRGNILNLNGAGDPLTPGYPANEYAYRRGIAEAVGLPSIPVHPIGYY

DAQKLLEKMGGSAPPDSSWRGSLKVPYNVGPGFTGNFSTQKVKMHIHSTN

EVTRIYNVIGTLRGAVEPDRYVILGGHRDSWVFGGIDPQSGAAVVHEIVR

FIGURE 14-7

```
                                                                    ----   XX
                                  **>)-----)))>****>**XXX-----XX
                                  ***>)-----)))>**XXX-----XX
410      420      430      440      450
SFGTLKKEGWRPRRTILFASWDAEEFGLLGSTEWAEENSRLLQERGVAYI

XXX****>>>****>-----*XXXXXXXXXXXX-----
XXX****>>>****>-----*XXXXXXXXXXXX-----
460      470      480      490      500
NADSSIEGNYTLRVDCTPLMYSLVHNLTKELKSPDEGFEGKSLYESWTKK

----**-->>-----XXXXXXXXX**XXXXXXXXXXXXXXX>>>*
----**-->>-----XXXXXXXXX**XXXXXXXXXXXXXXX>>>*
510      520      530      540      550
SPSPEFSGMPRISKLGSGNDFEVFFQRLGIASGRARYTKNWETNKFSGYP

****>-----****-----XXXXX>)***-----)>>)->>>>*
****>-----****-----XXXXX>)***-----)>>)->>>>*
560      570      580      590      600
```

FIGURE 14-8

```
LYHSVYETYELVEKFYDPMFKYHLTVAQVRGGMVFELANSIVLPFDCRDY
----XXXXXXXXXXXXXXXXX-X---------->XXX
----XXXXXXXXXXXXXX-X----------->XXX
    610   620   630   640   650

AVVLRKYADKIYSISMKHPQEMKTYSVSFDSLFSAVKNFTEIASKFSERL
XXXXXXXXX----X**XXXXX----------XXXXXXXXXXXXXXXXXXX
XXXXXXXXX----X**XXXXX----------XXXXXXXXXXXXXXXXXXXX
    660   670   680   690   700

QDFDKSNPIVLRMMNDQLMCLERAFIDPLGLPDRPFYRHVIYAPSSHNKY
XX>>>*----XXXXXXXXXXX--->>***>-------->****>
XX>>>*----XXXXXXXXXXX--->>***>-------->****>
    710   720   730   740   750

AGESFPGIYDALFDIESKVDPSKAWGEVKRQIYVAAFTVQAAAETLSEVA
------>---XXXXXXXX***XXXXXXX-------XXXXXXXXXXXXXXX
------>---XXXXXXXX***XXXXXXX-------XXXXXXXXXXXXXXX
```
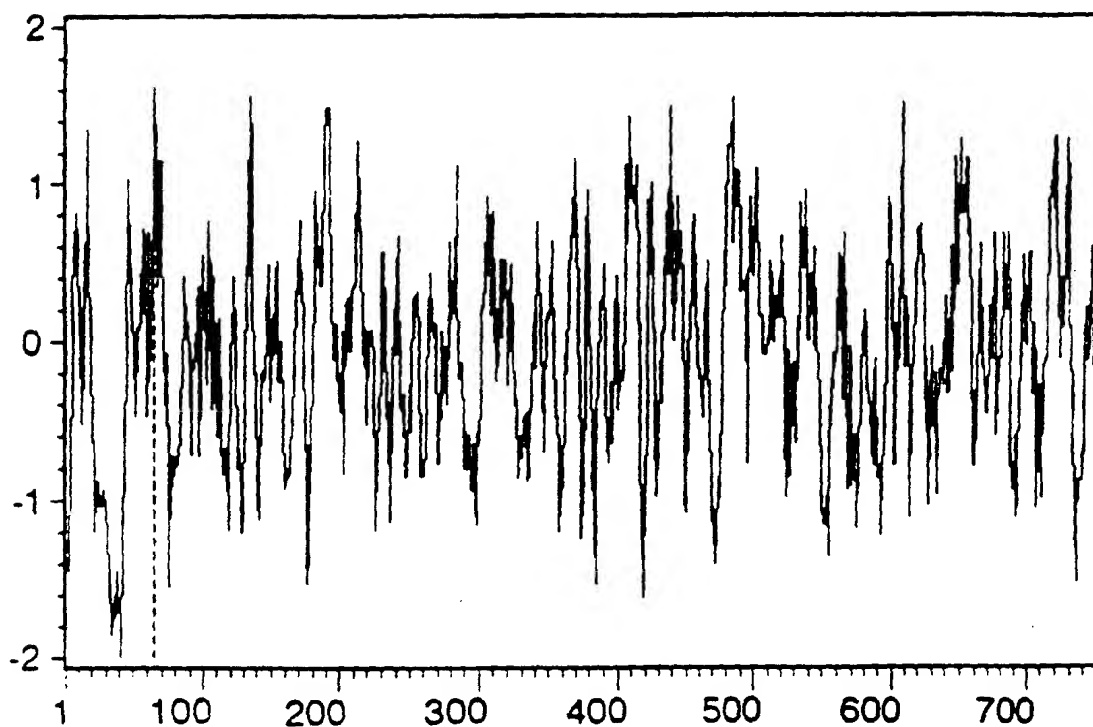
FIGURE 15A

FIGURE 15B

```
*** *** ******************************
* PREDICTION OF ANTIGENIC DETERMINANTS *
***** * * ******************************

Done on sequence PMSANTIGEN.
Total number of residues is: 750.
Analysis done on the complete sequence.

The method used is that of Hopp and Woods.
The averaging group length is: 6 amino acids.
-> This is the value recommended by the authors <-

----------------------------------------

The three highest points of hydrophilicity are:

( 1)  Ah= 1.62 : From    63 to    68 : Asp-Glu-Leu-Lys-Ala-Glu
( 2)  Ah= 1.57 : From   132 to   137 : Asn-Glu-Asp-Gly-Asn-Glu
( 3)  Ah= 1.55 : From   482 to   487 : Lys-Ser-Pro-Asp-Glu-Gly

Ah stands for: Average hydrophilicity.

Note that, on a group of control proteins, only the highest point was in 100%
of the cases assigned to a known antigenic group. The second and third points
gave a proportion of 33% of incorrect predictions.
```

FIGURE 16-1

```
The best scores are:                                        initn init1  opt
CHKTFER   G.gallus mRNA for transferrin receptor             203   120   321
RATTRFR   Rat transferrin receptor mRNA, 3' end.             164   164   311
HUMTFRR   Human transferrin receptor mRNA, complete cd       145   145   266


CHKTFER   G.gallus mRNA for transferrin receptor             203   120   321
51.9% identity in 717 nt overlap

            1020      1030      1040      1050      1060      1070
pmsgen   TGTCCAGCGTGGAAATATCCTAAATCTGAATGGTGCCAGGAGACCCTCTCACACCAGGTTA
                        :::  : :::::::: :::  :::::  :
CHKTFE   TACACTTATCCCATTCGGACACATGCCCACCTTGGAACTGGAGACCCTTACACCCCAGGCTT
            990       1000      1010      1020      1030      1040

            1080      1090      1100      1110      1120      1130
pmsgen   CCCAGCCAAATGAATATGCTTATAGGCGTGGAATTGCAGAGGCTGTGTTGGTCTTCCAAGTAT
         ::: :  : ::  :  : :: :: ::  :: ::  ::  ::
CHKTFE   CCCTTCGTTCAACCACACCCA---GTTTCCACCAGTTGAATCTTCAGGACTACCCCACAT
            1050      1060      1070      1080      1090      1100

            1140      1150      1160      1170      1180      1190
pmsgen   TCCTGTTCATCCAATTGGATACTATGATGCCACAGAAGCTCCTAGAAAAAATGGGTGGCTC
         : :::::::  : ::  : :::::: :::
CHKTFE   TGCTGTTCAGACCATCTCTAGCCAGTGCAGCCAGCCAGGCTGTTCAGCAAAATGGATGGAGA
            1110      1120      1130      1140      1150      1160
```

FIGURE 16-2

```
             1200      1210      1220      1230      1240      1250
pmsgen  AGCACCACCAGATAGCAGCTGGAGAGGAAGTCTCAAAGTGCCCTACAATGTTGGACCTGG
             ::    :  ::  ::  :  ::::  :::     :::  ::  :: :::  :: :::
CHKTFE  CACATGCTCTGA-AG--GTTGGAAGGTGCCATCCA---TTCCTGTAGGT--GAC--AA
             1170      1180      1190      1200             1210

             1260      1270      1280      1290      1300      1310
pmsgen  CTTTACTGGAAACTTTTCTACACAAAAGTCAAGATGCACATCCACTCTACCAATGAAGT
             :  :  :  :     :::   ::  ::  :::  :  :::    :::
CHKTFE  CAAAGCCAGGAGA---GCCAGA-TAATGGTGAAACTAGATGTGAACAATTCCATGAAAGA
             1220      1230      1240      1250      1260

             1320      1330      1340      1350      1360      1370
pmsgen  GACAAGAATTTACAATGTGATAGGTACTCTCAGAGGAGCAGTGGAACCAGACAGATATGT
             :  ::  :   :::  ::  :  :::::  ::  :  :::::  :  :::::
CHKTFE  CAGGAAGATTCTGAACATCTTCGGTGCTATCCAGGGATTTGAAGAACCTGATCGGTATGT
             1270      1280      1290      1300      1310      1320

             1380      1390      1400      1410      1420      1430
pmsgen  CATTCTCTGGGAGGTCACCGGGACTCATGGGTGTGTTTGGTGGTATTGACCCTCAGAGTGGGAGC
             :  : ::::  ::::  :::::  ::  :  :::  :  :  ::  :  :::::  :
CHKTFE  TGTGATTGGAGCCCAGAGAGACTCCTGGGGCCCAGGAGTGGCTAAAGCTGGCCACTGGGAAC
             1330      1340      1350      1360      1370      1380
```

FIGURE 16-3

```
                1440      1450      1460      1470      1480      1490
pmsgen   AGCTGTTGTTCATGAAATTGTGAG---GAGCTTTGGAACACTGAAAAGGAAGGGTGGAG
         ::: :  : ::: :::    :  :: :: :   : ::::::: :: :: :: :
CHKTFE   TGCTATATTGTTGGAACTTGCCCGTGTGATCTCAGACATAGTGAAAAACGAGGGCTACAA
                1390      1400      1410      1420      1430      1440


                1500      1510      1520      1530      1540      1550
pmsgen   ACCTAGAAGAACAATTTGTTTGCAAGCTGGGATGCAGAGAATTTGGTCTTCTTGGTTC
         ::: :  : ::: :  : X::: :::: ::::: :: : : ::: :: :
CHKTFE   ACCGAGGCGAAGCATCATCTTTGCTAGCTGGGAGTGCAGGAGACTACCGGAGCTGTGGGTGC
                1450      1460      1470      1480      1490      1500


                1560      1570      1580      1590      1600      1610
pmsgen   TACTGAGTGGGCAGAGGAGAATTCAAGACTCCTTCAAGAGCCGGTGGCTTATATTAA
         :::::: :::  :::: : :X  : :: ::  : : :::: :: :
CHKTFE   TACTGAATGGCTGGAGGGGGTACTCTGCCCATGCTGCCAAAGCTTTCACTTACATCA-
                1510      1520      1530      1540      1550      1560


                1620      1630      1640      1650      1660      1670
pmsgen   TGC-TGACTCATCTATAGAAGGAAAACTA-CACTCTGAGAGTTGATTGTACACCGCTGATG
         :: ::  :  :: ::  :: :: ::: :: :  : :: : : :: :: ::
CHKTFE   -GCTTGGGATGCTCCAGTCCTGGGAGCAAGCCATGTCAAGATTTCTGCCAGCCCCTTGCTG
                1570      1580      1590      1600      1610      1620
```

FIGURE 16-4

```
              1680      1690      1700      1710      1720      1730
pmsgen TACAGCTTGGTACACAACCTAACAAAGAGCTGAAAAGCCCTGATGAAGGCTTTGAAGGC
       :: :  :: ::  :  :  :  : :::  : :: :: :: :: ::
CHKTFE TATATGCTGCTGGGGAGTATTATGAAGGGGTGAAGAATCCAGCAGTCTCAGAGAGC
              1630      1640      1650      1660      1670      1680


              1740      1750      1760      1770      1780      1790
pmsgen AAATCTCTTTATGAAAGTTGGACTAAAAAAGTCCTTCCCCAGAGTTCAGTGGCATGCCC
       ::: :: :: :::: :: :
CHKTFE ----CTCTATAACAGACTTGGCCCCAGACTGGGTAAAAGCAGTTGTTCCTCTTGGCCCTGGA
              1690      1700      1710      1720      1730
```

FIGURE 16-5

```
RATTRFR   Rat transferrin receptor mRNA, 3' end.              164  164  311
          55.5% identity in 560 nt overlap

                1210      1220      1230      1240      1250
pmsgen  CCACCAGAGATAGCCAGCTGGAGAGGAAGTCTCAAAGTGCCCTACAATGTTGGACCTGGCTT-
                                      :: :: ::: :: :: : : : ::  : ::
RATTRF  TGCAGAAAAGCTATTCAAAAACATGGAAGGAAACTGTCCTCCTAGTTGGAATATAGATTC
          610       620       630       640       650       660

                1260      1270      1280      1290      1300      1310
pmsgen  -TACTGGAAACTTTTCTACACAAAAGTCAAGATGCACATC-CACTCT-ACCAATG----
         : :: :: :: ::        :: : : ::::: ::: : : :::: : : ::::
RATTRF  CTCATGTGTAAGCTGGAACTTTCACAGAATCAAAATGTGAAGCTCACTGTGAACAATGTACT
          670       680       690       700       710       720
```

FIGURE 16-6

```
                 1320      1330      1340      1350      1360      1370
pmsg n  --AAGTGACAAGAATTTACAATGTGATAGGTACTCTCAGAGGAGCAGTGGAACCAGACAG
          :: ::::::::: ::  :  :  ::: :  :::::::::::  :
RATTRF  GAAAGAAACAAGAATACTTAACATCTTTGGCGTTATTAAAGGCTATGAGGAACCAGACCG
            730       740       750       760       770       780

                 1380      1390      1400      1410      1420      1430
pmsgen  ATATGTCATTCTGGGAGGTCACCGGGACTCATGGGTGTGTTTGGTGGTATTGACCCTCAGAG
          ::  :  :  :::: ::   : :::::  ::::  ::  :   :::
RATTRF  CTACATTGTAGTAGGAGCCCAGAGACGCTTGGGCCCCTGGT-GTTGCGAAGTCCAGTG
            790       800       810       820       830       840

                 1440      1450      1460      1470      1480
pmsgen  T-GGAGCAGCTGTGTTGTTCATGAAAATTGTGAGGAGCTTTGGAACA-CTGA---AAAAGGAA
          : ::: ::: :: : ::::: :  :: : ::  ::
RATTRF  TGGGAACAGGTCTT-CTGTTGAAACTGCCCAGTATTCTCAGATATGATTCAAAAGAT
            850       860       870       880       890       900

                 1490      1500      1510      1520      1530      1540
pmsgen  GGGTGGAGACCTAGAAGAACAATTTGTTGCAAGCTGGGATGCAGAAGAATTTGGTCTT
          :: : X:::: ::  ::  :::::: :::::  ::: : :::  :
RATTRF  GGATTTAGACCCAGCAGGAGTATTATCTTTGCCAGCTGGACTGCCAGGAGACTATGGAGCT
            910       920       930       940       950       960
```

FIGURE 16-7

```
                  1550      1560      1570      1580      1590      1600
         pmsgen  CTTGGTTCTACTGAGTGGGCAGAGGAGAA---TTCAAGACTCCTTCAAGAGCGTGGCGTG
                 ::::: : ::::::::::     :: :: :: :: : ::          : :
         RATTRF  GTTGGTCCGACTGGCTGGAGTGGGTACCTTTCATCTTTGCATCTAAAG---GCTTTC
                  970       980       990       1000      1010      1020

                  1610      1620      1630      1640      1650      1660
         pmsgen  GCTTATATTAATGCTGACTCATCTATAGAAGGAAACTA-CACTCTGAGAGTTGATTGTAC
                 :::: ::::::: :::   :  :: ::::: ::  : :::  : ::: :: :
         RATTRF  ACTTACATTAAT-CTGGATAAAGTCGTCCTGGTACTAGCAACTTCAAGGTTTCTGCCAG
                  1030      1040      1050      1060      1070      1080

                  1670      1680      1690      1700      1710      1720
         pmsgen  ACCGCTGATGTACAGCTTGGTACACAACCTAACAAAGAGCTGAAAAGC-CCTGATGAAG
                 :: :: : ::: :  :  :  :: ::: : :: : :::: : ::
         RATTRF  CCCCCTATTATATACACTTATGGGAAGATAATGCCAGGA--CGTAAAGCATCCGA-----
                  1090      1100      1110      1120      1130
```

FIGURE 16-8

```
                        1730      1740          1750      1760      1770
pmsgen  GCTTTGAAGGCAAATCTCTTTAT-GAA-----AGTTGGACTAAAAAAGTCCTTCCCCAG
                   :::: :: ::::: ::: ::: :::  :   : ::::: :: ::::: :
RATTRF  ---TTGATGGAAAATATCTATATCGAAACAGTAATTGGATTAGCAAAATTGAGGAACTTT
                   1140      1150      1160      1170      1180      1190

                        1780      1790      1800      1810      1820      1830
pmsgen  AGTTCAGTGGCATGCCCAGGATAAGCAAATTGGGATCTGGAAATGATTTGAGGTGTTCT

RATTRF  CCTTGGACAATGCTGCATTCCCTTTCTTGCATATTCAGGAATCCCAGCCAGTTTCTTTCT
                   1200      1210      1220      1230      1240      1250
```

FIGURE 16-9

HUMTFRR Human transferrin receptor mRNA, complete cd 145   145   266
54.3% identity in 464 nt overlap

```
                   1230      1240      1250      1260      1270
pmsg n AGGAAGTCTCAAAGTGCCCTACAATGTTGGACCTGGCTTTAC-TGGAAACTTTCTACAC
                                               : : :: ::: :: : .: .: ::
HUMTFR TATGGAAGGAGACTGTCCCTCTGACTGGAAAACAGACTCTACACATGTAGGATGGTAACCTC
       1140      1150      1160      1170      1180      1190

       1280      1290      1300      1310      1320      1330
pmsgen AAAAAGTCAAGATGCACATC-CACTCT-ACCAATG------AAGTGACAAGAATTTACAA
       : :::: : ::: :: : ::::: :: : ::::       ::: :: :: ::::: ::
HUMTFR AGAAAGCAAGAATGTGAAGCTCACTGTGCTGAGCAATGTGCTGAAAGAGATAAAAATTCTTAA
       1200      1210      1220      1230      1240      1250

                   1340      1350      1360      1370      1380      1390
pmsg n TGTGATAGGTACTCTCAGAGGAGCAGTGGAACCAGACCAGATATGTCATTCTCGGGAGGTCA
       : : :: : ::: : : :::   :: :::::::::  : :: : :: ::::: : :
HUMTFR CATCTTTGGGAGTTATTAAAGGCTTTGTAGAACCAGATCACTATGTTGTGTAGTTGGGGCCCA
       1260      1270      1280      1290      1300      1310

            1400      1410      1420      1430      1440      1450
pmsgen CCGGGACTCATGGGTGTTTGGTGGTATTGACCCTCAGAGT-GGAGCAGCTGTTGTTCATG
       : :: :::::::: ::: :  : : :: :: ::::: : ::
HUMTFR GAGAGATGCCATGGGCCCCTGGAGCTGCAAAATC-CGGTGTAGGCACAGCTCTCCTATTGA
       1320      1330      1340      1350      1360      1370
```

FIGURE 16-10

```
                    1460      1470      1480      1490      1500
pmsg n  AAATTG---TGAGGAGCTTTGGAACACTGAAAAAGGAAGGGTGGAGACCTAGAAGAACAA
        :: :::  :: :: :  :  : :: :: X:::  :: :: :::: ::
HUMTFR  AACTTGCCCAGATGTTCTCAGATATGGTCTTAAAGATGGGTTTCAGCCCAGCAGAAGCA
        1380      1390      1400      1410      1420      1430

        1510      1520      1530      1540      1550      1560
pmsgen  TTTTGTTTGCAAGCTGGGATGCAGAAGAATTTGGTCTTCTTGGTCTACTGAGTGGGCAG
        :: : ::::: :: ::: :: ::: :: :::::   ::::: : ::::: ::: ::
HUMTFR  TTATCTTGCCAGTGTTGGAGTGCTGGAGTGCTTGGATCGGTTGGTGCCACTGAATGGCTAG
        1440      1450      1460      1470      1480      1490

        1570      1580      1590      1600      1610      1620
pmsgen  A-GGAGAATTCAAGACTCCTTCAAGAGCCGTGGCGTGGCTTATATTAATGCTGACTCATCT
        : ::: :  : : ::: ::           :  ::::::::X  ::  :  ::
HUMTFR  AGGGATACCTTTCGTC-CCTGCATTTAAAGGCTTTCACTTAAAGGCTTTATATTAATCTGGATAAAGCG
        1500      1510      1520      1530      1540      1550

        1630      1640      1650      1660      1670      1680
pmsgen  ATAGAAGGAAACTACACTCTGAGAGTTGATTGTACACCGCTGATGTACA-GCTTGGT-AC
        :   :: :: ::  :  :: :: : :  :: ::  :::: : :::: ::  ::
HUMTFR  GTTCTTGGTACCAGCAACTTCAAGGTTTCTGCCAGCCCCACTGTTGTATACGCTTATTGAG
        1560      1570      1580      1590      1600      1610
```

FIGURE 16-11

```
                 1690      1700      1710      1720      1730      1740
pmsg n  ACAACCTAACAAAAGAGCTGAAAAGCCCTGATGAAGGCTTTGAAGGCAAATCTCTTATG
        : :::  :::::::
HUMTFR  AAAACAATGCAAAATGTGAAGCCATCCGGTTACTGGGCAATTCTATATCAGGACAGCAAC
                 1620      1630      1640      1650      1660      1670
```
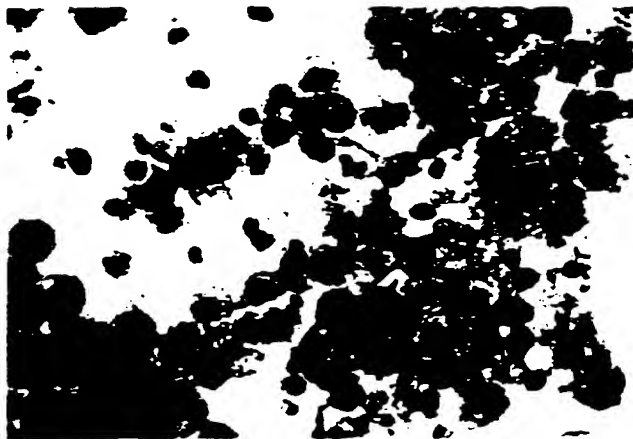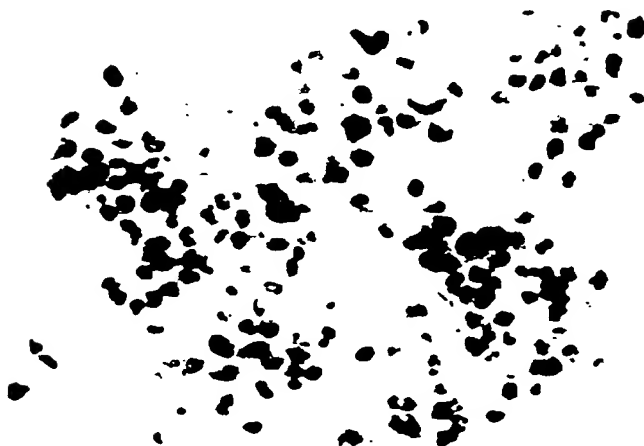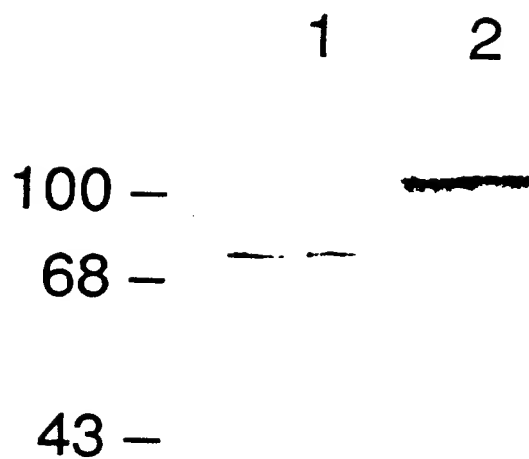
FIGURE 17A

FIGURE 17B

FIGURE 17C

FIGURE 18

FIGURE 19

FIGURE 20

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

400

350
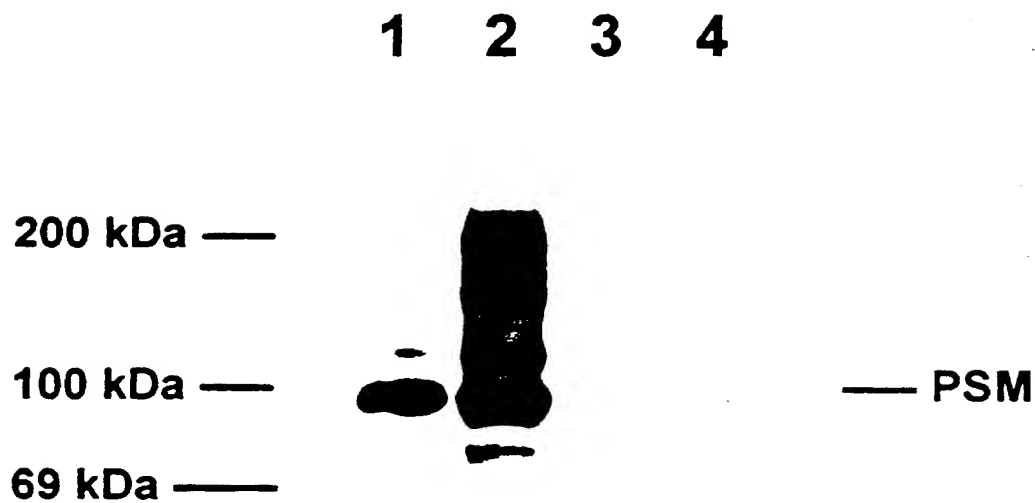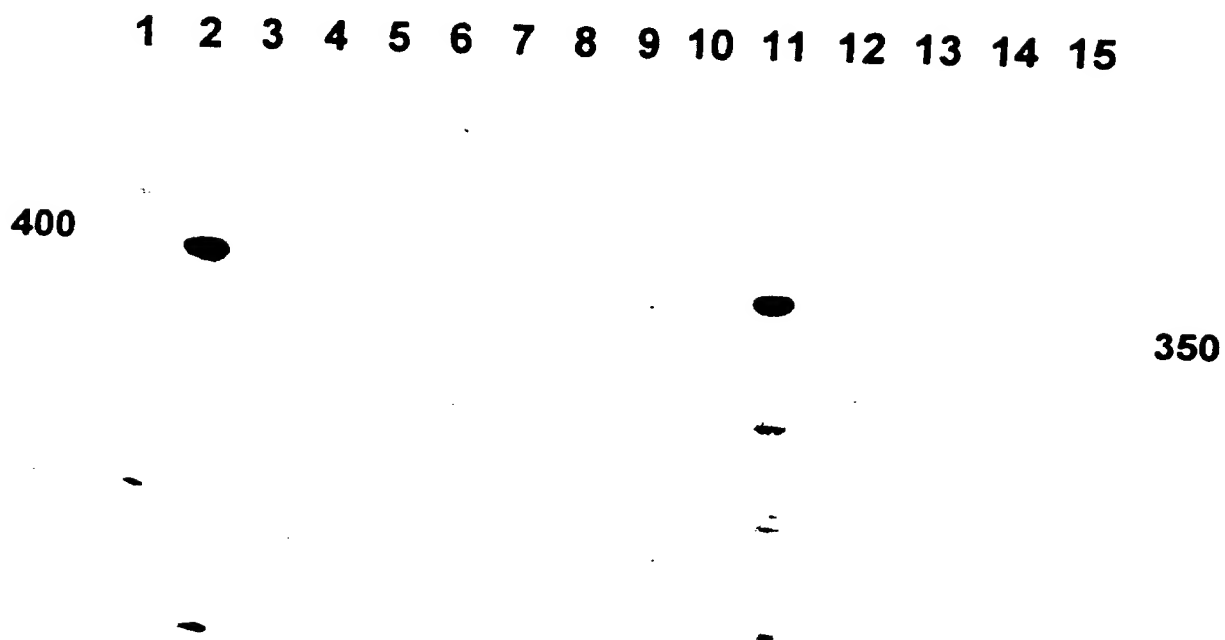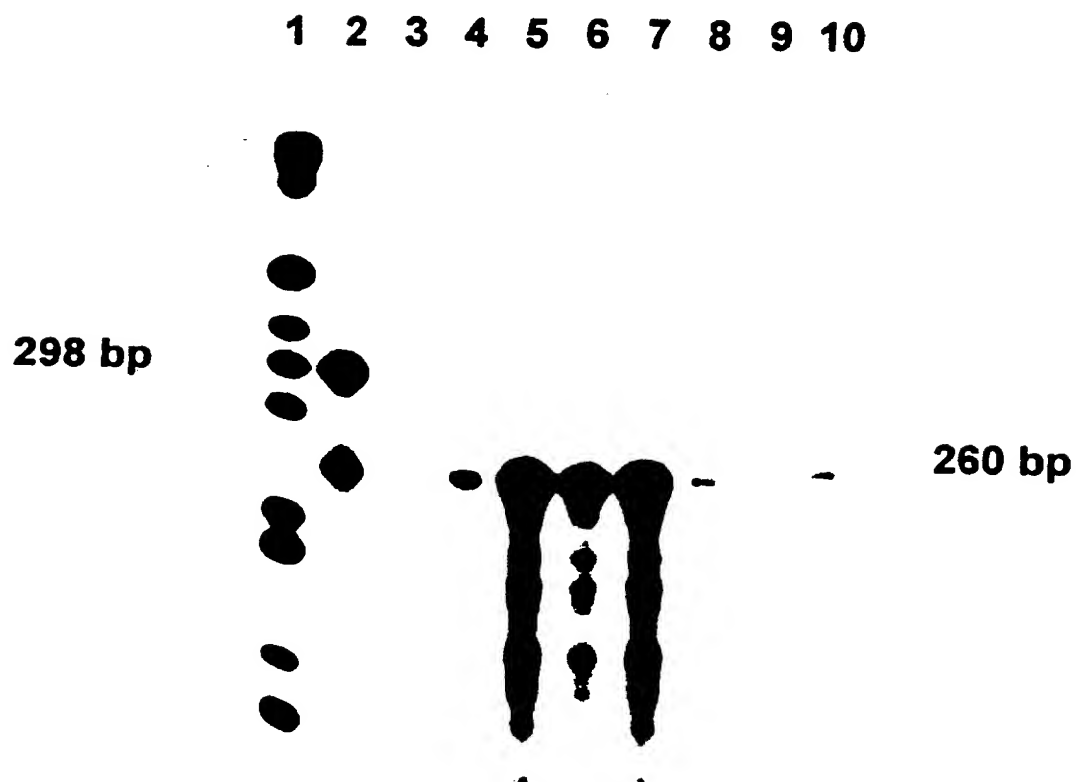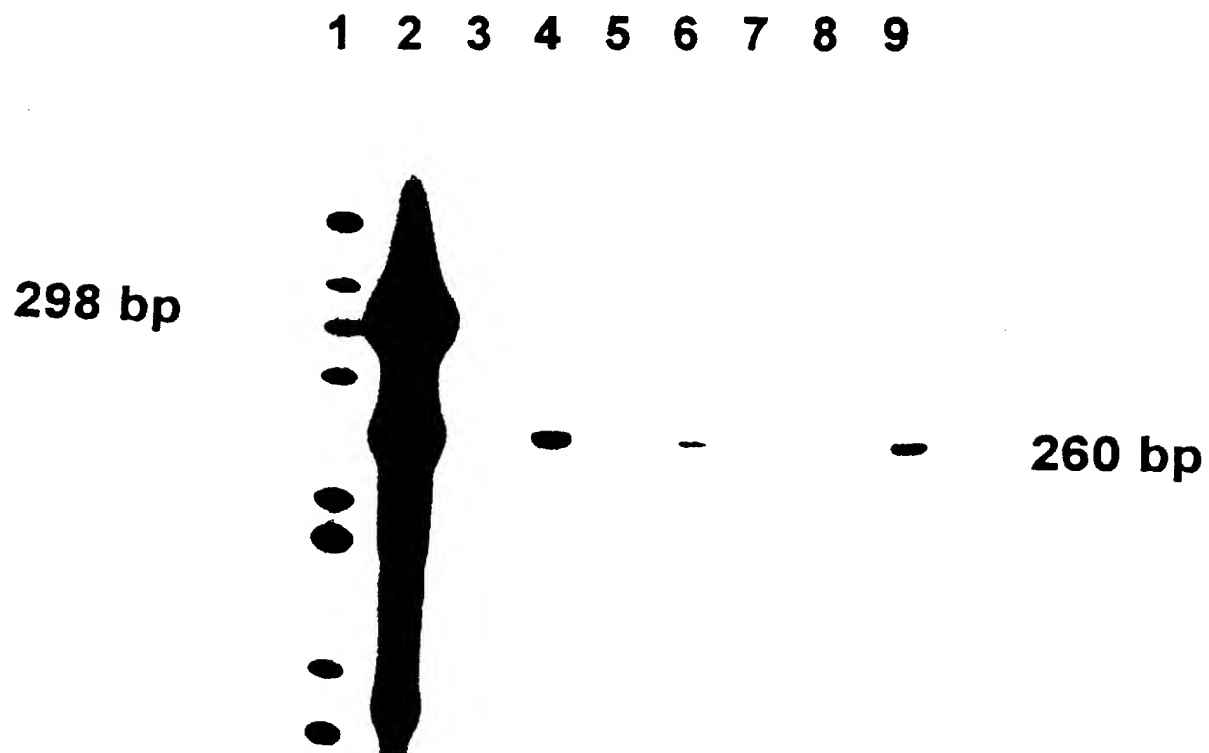
FIGURE 21

FIGURE 22

FIGURE 23

CELL LINE/TYPE	11p11.2-13 REGION	METASTATIC	PSM RNA DETECTED	PSM DNA DETECTED
LNCap			+ +	ND
HUMAN PROSTATE			+ +	ND
A9 (FIBROSARCOMA)	NO	NO	−	−
A9(11) (A9+HUM. 11)	YES	NO	−	REPEAT
AT6.1 (RAT PROSTATE)	NO	YES	−	−
AT6.1-11-c11	YES	NO	+	+ +
AT6.1-11-c12	NO	YES	−	−
R1564 (RAT MAMMARY)	NO	YES	−	−
R1564-11-c14	YES	YES	−	+
R1564-11-c15	YES	YES	−	REPEAT
R1564-11-c16	YES	YES	−	ND
R1564-11-c12	YES	YES	ND	+
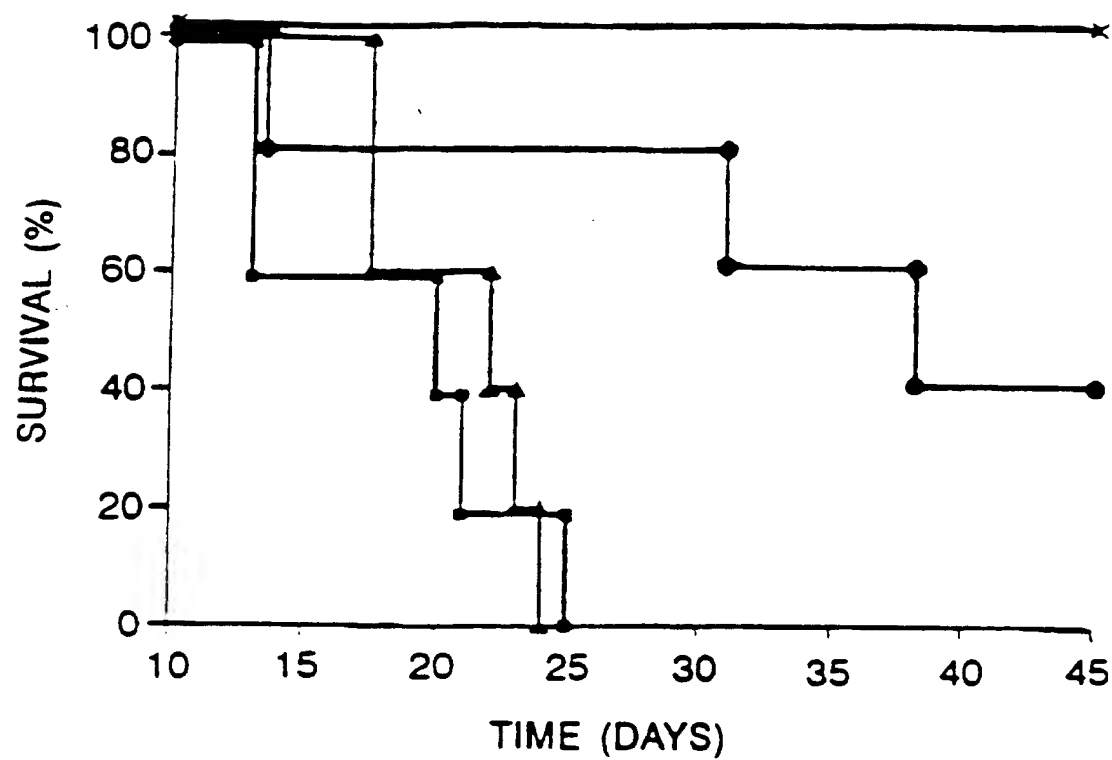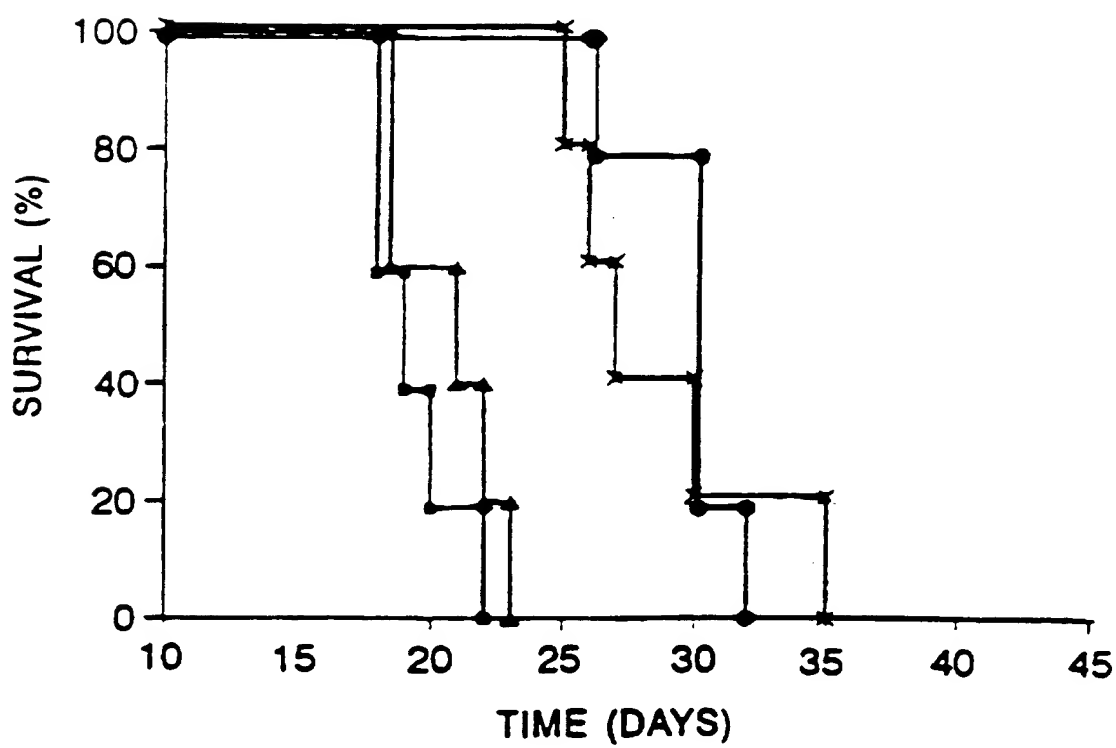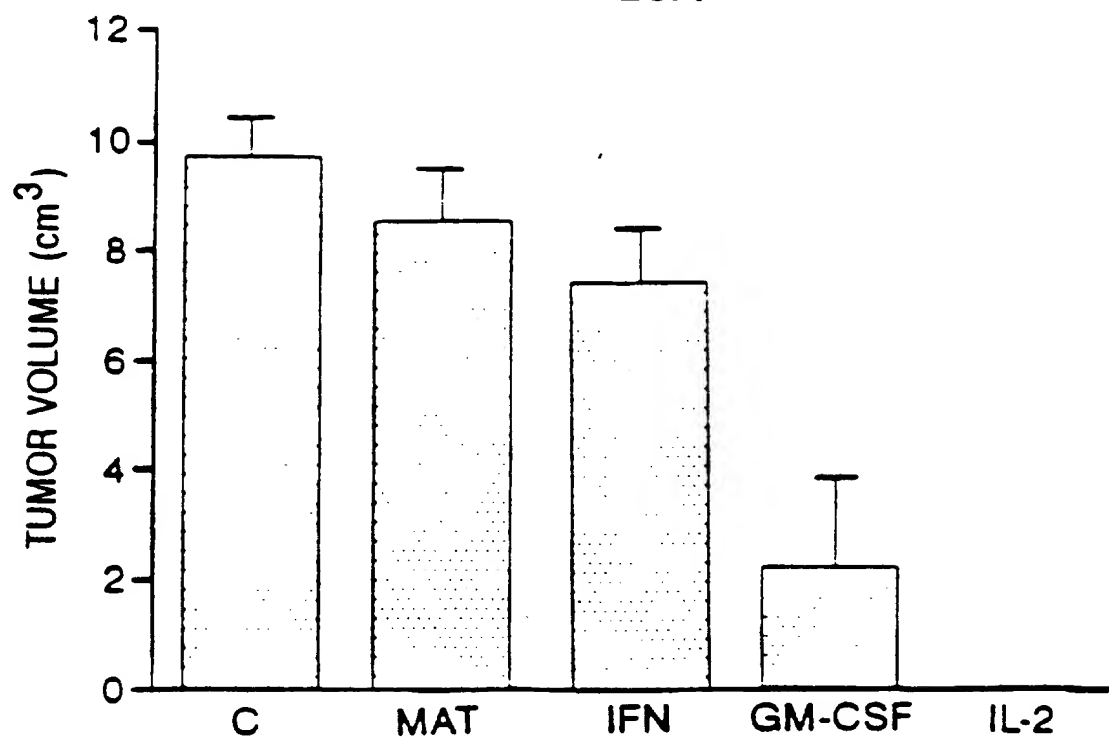
FIGURE 24A

TIME (DAYS)

FIGURE 24B

TIME (DAYS)

FIGURE 25A

FIGURE 25B
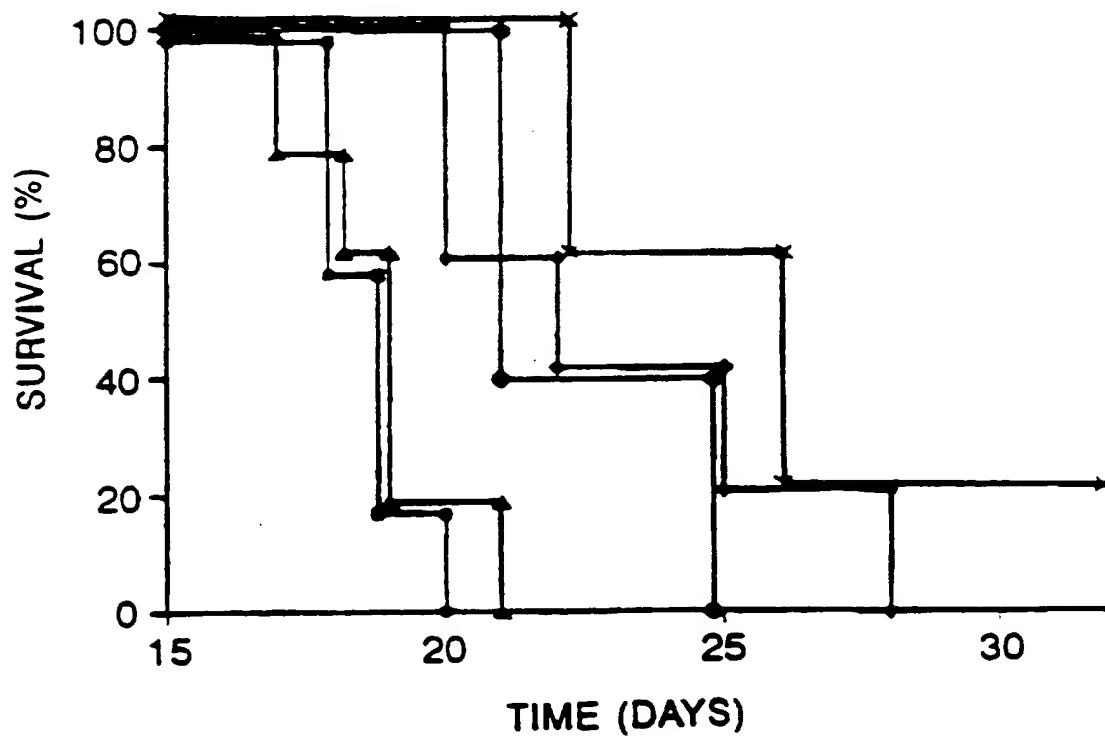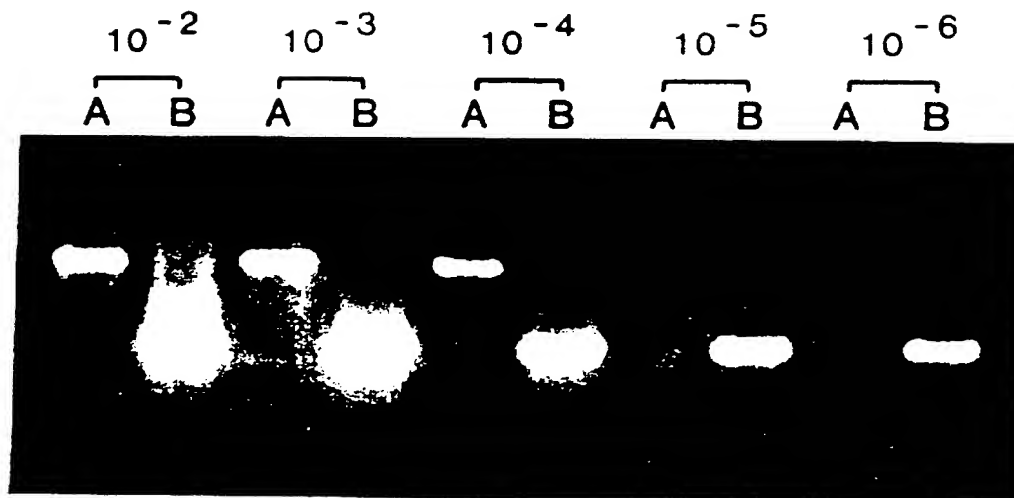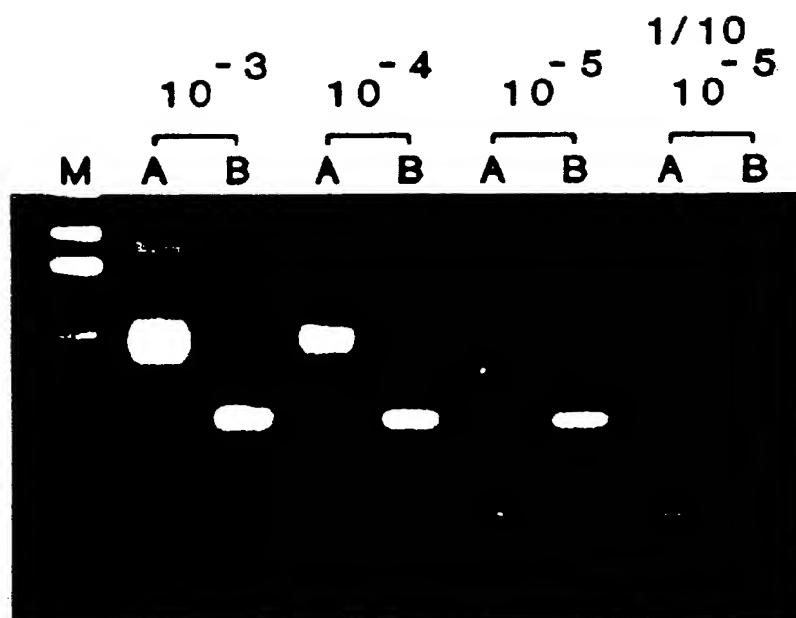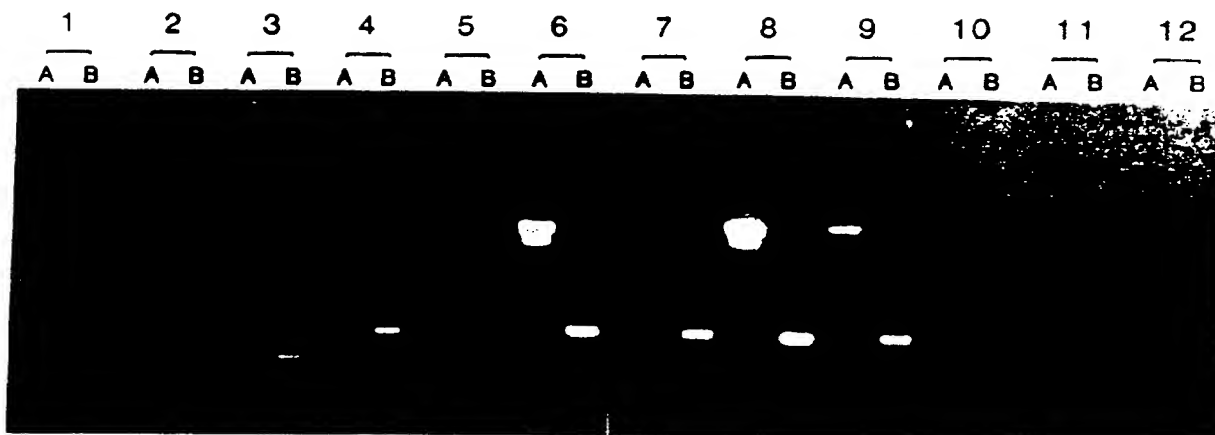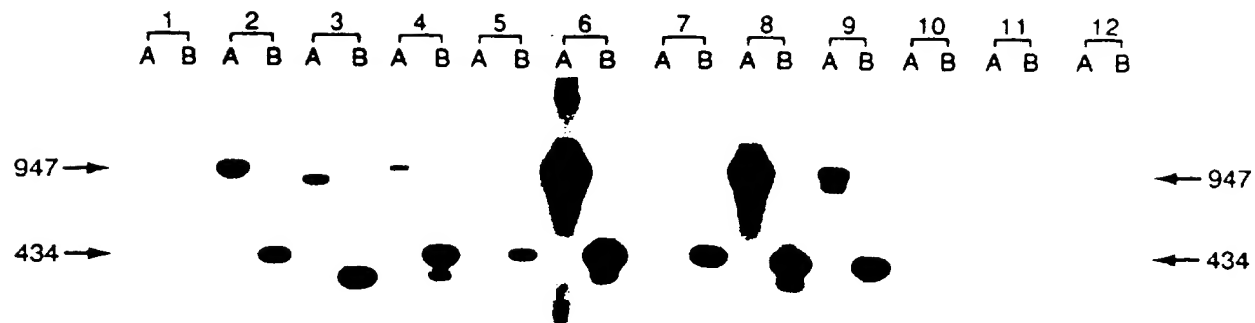
FIGURE 26

FIGURE 27

FIGURE 28

FIGURE 29

FIGURE 30

Patient	Stag	Treatment	PSA	PAP	PSA-PCR	PSM-PCR
1	T2NxMo	None	8.9	0.7	−	+
2	T2NoMo	RRP 7/93	6.1	−	−	+
3	T2CNoMo	PLND 5/93	4.5	0.1	−	+
4	T2BNoMo	RRP 3/92	NMA	0.4	−	+
5	T3NxMo	Proscar + Flutamide	51.3	1.0	−	+
6	Recur T3	I-125 1986	54.7	1.4	−	+
7	T3ANoMo	RRP 10/92	NMA	0.3	−	+
8	T3NxMo	XRT 1987	7.5	0.1	−	−
9	T3NxMo	Proscar + Flutamide	35.4	0.7	−	−
10	D2	S/P XRT Flutamide +Emcyt	311	4.5	+	+
11	D2	RRP 4/91 Lupron 10/92 Velban + Emcyt 12/92	1534	1.4	+	+
12	T2NoMo	RRP 8/91	NMA	0.5	−	+
13	T3NoMo	RRP 1/88 Lupron + Flutamide 5/92	0.1	0.3	−	−
14	D1	PLND 1989 XRT 1989	1.6	0.4	−	−
15	D1	Proscar + Flutamide	20.8	0.5	−	−
16	T2CNoMo	RRP 4/92	0.1	0.3	−	−

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FIGURE 31A

```
              10          20          30          40          50          60
               |           |           |           |           |           |
     1 AAGGGTGCTC CTTAGGCTGA ATGCTTGCAG ACAGGATGCT TGGTTACAGA TGGGCTGTGA
       TTCCCACGAG GAATCCGACT TACGAACGTC TGTCCTACGA ACCAATGTCT ACCCGACACT


    61 CTCGAGTGGA GTTTTATAAG GGTGCTCCTT AGGCTGAATG CTTGCAGACA GGATGCTTGG
       GAGCTCACCT CAAAATATTC CCACGAGGAA TCCGACTTAC GAACGTCTGT CCTACGAACC


   121 TTACAGATGG GCTGTGAGCT GGGTGCTTGT AAGAGGATGC TTGGGTGCTA AGTGAGCCAT
       AATGTCTACC CGACACTCGA CCCACGAACA TTCTCCTACG AACCCACGAT TCACTCGGTA


   181 TTGCAGTTGA CCCTATTCTT GGAACATTCA TTCCCCTCTA CCCCTGTTTC TGTTCCTGCC
       AACGTCAACT GGGATAAGAA CCTTGTAAGT AAGGGGAGAT GGGGACAAAG ACAAGGACGG


   241 AGCTAAGCCC ATTTTTCATT TTTCTTTTAA CTCCTTAGCG CTCCGCAAAA CTTAATCAAT
       TCGATTCGGG TAAAAAGTAA AAAGAAAATT GAGGAATCGC GAGGCGTTTT GAATTAGTTA


   301 TTCTTTAAAC CTCAGTTTTC TTATCTGTAA AAGGTAAATA ATAATACAGG GTGCAACAGA
       AAGAAATTTG GAGTCAAAAG AATAGACATT TTCCATTTAT TATTATGTCC CACGTTGTCT


   361 AAAATCTAGT GTGGTTTACA TAATCACCTG TTAGAGATTT TAAATTATTT CAGGATAAGT
       TTTTAGATCA CACCAAATGT ATTAGTGGAC AATCTCTAAA ATTTAATAAA GTCCTATTCA


   421 CATGATAATT AAATGAAATA ATGCACATAA AGCACATAGT GTGGTGTCCT CCATATAGAA
       GTACTATTAA TTTACTTTAT TACGTGTATT TCGTGTATCA CACCACAGGA GGTATATCTT


   481 AATGCTCAGT ATATTGGTTA TTAACTACTT GTTGAAGGTT TATCTTCTCC ACTAAACTGT
       TTACGAGTCA TATAACCAAT AATTGATGAA CAACTTCCAA ATAGAAGAGG TGATTTGACA


   541 AAGTTCCACA AGCCTTACAA TATGTGACAG ATATTCATTC ATTGTCTGAA TTCTTCAAAT
       TTCAAGGTGT TCGGAATGTT ATACACTGTC TATAAGTAAG TAACAGACTT AAGAAGTTTA


   601 ACATCCTCTT CACCATAGCG TCTTATTAAT TGAATTATTA ATTGAATAAA TTCTATTGTT
       TGTAGGAGAA GTGGTATCGC AGAATAATTA ACTTAATAAT TAACTTATTT AAGATAACAA


   661 CAAAAATCAC TTTTATATTT AACTGAAATT TGCTTACTTA TAATCACATC TAACCTTCAA
       GTTTTTAGTG AAAATATAAA TTGACTTTAA ACGAATGAAT ATTAGTGTAG ATTGGAAGTT


   721 AGAAAACACA TTAACCAACT GTACTGGGTA ATGTTACTGG GTGATCCCAC GTTTTACAAA
       TCTTTTGTGT AATTGGTTGA CATGACCCAT TACAATGACC CACTAGGGTG CAAAATGTTT
```

FIGURE 31B

781 TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGGTAATCA GCTTGGACAG
    ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGGT CCCCATTAGT CGAACCTGTC

841 GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
    CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGGTCACG

901 CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAGT
    GTGTTCGTTT GAGGTATTTC CATAGGACAC GACTTATCTC TGACATCTCA CCATGTTTCA

961 AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
    TTCTGTCTGT AATATAATTC AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021 AAATTTCAGT TTTACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
     TTTAAAGTCA AAATGGTACA CATTTAGTCC TTCTCATTAT CTTGTTTGGA ACTTCCCAGG

1081 CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
     GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141 AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
     TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTTATCCTT GTAATCCTTT

1201 GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATTCCCTTC CTTCCTGCCC
     CTATATCATG TAAGTCCTAA AACAATCTTT CTCTACTTCT TTAAGGGAAG GAAGGACGGG

1261 TAGGTCATCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTTCA
     ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAAAGGG TTTAAAAAGT

1321 CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTC
     GAAACGAGTC TTTCAGATGT AGCTTCGTGG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381 CACTTAACTC ATACTGTGCT CTCCCTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
     GTGAATTGAG TATGACACGA GAGGGAAAGA GTTTCGTTTG ACAAACGATA AGGAACTTAT

1441 CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGCCCCTAAT GTTTCTTCTC
     GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGGTA CCGGGGATTA CAAAGAAGAG

1501 ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTTCT GTTAAAAGCA GTGCTTCCAT
     TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTTCGT CACGAAGGTA

1561 AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA

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FIGURE 31C

```
      TTTCATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTTGTGT CAAATAAAAT


1621  TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
      ATTTCGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATAATTCT TAAATATCGT


1681  GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
      CCCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTTCTAACT AAAATATAGA


1741  ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
      TAATGCATTC TGTCATCGGT CTGTATCGGC CCTATACTTT TATTTCAGAG ACGGAAGTTG


1801  AAGTTCCAGT ATTCTTTTCT TTCCTCCCCT CCCCTCCCCT CCCTTCCCCT CCCCTTCCTT
      TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAAGGGGA GGGGAAGGAA


1861  CCCTTTCCCT TCCCTTCCTT TCTTTCTTGA GGGAGTCTCA CTCTGTCACC AGGCTCCAGT
      GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCCTCAGAGT GAGACAGTGG TCCGAGGTCA


1921  GCAGTGGCGC TATCTTGGCT GACTGCAACC TCCGCCTCCC CGGTTCAAGC GATTCTCCTG
      CGTCACCGCG ATAGAACCGA CTGACGTTGG AGGCGGAGGG GCCAAGTTCG CTAAGAGGAC


1981  CCTCAGCCTC CTGAGTAGCT GGGACTACAG GAGCCCGCCA CCACGCCCAG CTAATTTTTG
      GGAGTCGGAG GACTCATCGA CCCTGATGTC CTCGGGCGGT GGTGCGGGTC GATTAAAAAC


2041  TATTTTTAGT AGAGATGGGG TTTCACCATG TTGGCCAGGA TGGTCTCGAT TTCTCGACTT
      ATAAAAATCA TCTCTACCCC AAAGTGGTAC AACCGGTCCT ACCAGAGCTA AAGAGCTGAA


2101  CGTGATCCGC CTGTCTGGGC CTCCCAAAGT GCTGGGATTA CAGGCGTGAG CCACCACGCC
      GCACTAGGCG GACAGACCCG GAGGGTTTCA CGACCCTAAT GTCCGCACTC GGTGGTGCGG


2161  CGGCTTTAAA AAATGGTTTT GTAATGTAAG TGGAGGATAA TACCCTACAT GTTTATTAAT
      GCCGAAATTT TTTACCAAAA CATTACATTC ACCTCCTATT ATGGGATGTA CAAATAATTA


2221  AACAATAATA TTCTTTAGGA AAAAGGGCGC GGTGGTGATT TACACTGATG ACAAGCATTC
      TTGTTATTAT AAGAAATCCT TTTTCCCGCG CCACCACTAA ATGTGACTAC TGTTCGTAAG


2281  CCGACTATGG AAAAAAAGCG CAGCTTTTTC TGCTCTGCTT TTATTCAGTA GAGTATTGTA
      GGCTGATACC TTTTTTTCGC GTCGAAAAAG ACGAGACGAA AATAAGTCAT CTCATAACAT


2341  GAGATTGTAT AGAATTTCAG AGTTGAATAA AAGTTCCTCA TAATTATAGG AGTGGAGAGA
      CTCTAACATA TCTTAAAGTC TCAACTTATT TTCAAGGAGT ATTAATATCC TCACCTCTCT
```

FIGURE 31D

2401 GGAGAGTCTC TTTCTTCCTT TCATTTTTAT ATTTAAGCAA GAGCTGGACA TTTTCCAAGA
     CCTCTCAGAG AAAGAAGGAA AGTAAAAATA TAAATTCGTT CTCGACCTGT AAAAGGTTCT

2461 AAGTTTTTTT TTTTTAAGGC GCCTCTCAAA AGGGGCCGGA TTTCCTTCTC CTGGAGGCAG
     TTCAAAAAAA AAAAATTCCG CGGAGAGTTT TCCCCGGCCT AAAGGAAGAG GACCTCCGTC

2521 ATGTTGCCTC TCTCTCTCGC TCGGATTGGT TCAGTGCACT CTAGAAACAC TGCTGTGGTG
     TACAACGGAG AGAGAGAGCG AGCCTAACCA AGTCACGTGA GATCTTTGTG ACGACACCAC

2581 GAGAAACTGG ACCCCAGGTC TGGAGCGAAT TCCAGCCTGC AGGGCTGATA AGCGAGGCAT
     CTCTTTGACC TGGGGTCCAG ACCTCGCTTA AGGTCGGACG TCCCGACTAT TCGCTCCGTA

2641 TAGTGAGATT GAGAGAGACT TTACCCCGCC GTGGTGGTTG GAGGGCGCGC AGTAGAGCAG
     ATCACTCTAA CTCTCTCTGA AATGGGGCGG CACCACCAAC CTCCCGCGCG TCATCTCGTC

2701 CAGCACAGGC GCGGGTCCCG GGAGGCCGGC TCTGCTCGCG CCGAGATGTG GAATCTCCTT
     GTCGTGTCCG CGCCCAGGGC CCTCCGGCCG AGACGAGCGC GGCTCTACAC CTTAGAGGAA

2761 CACGAAACCG ACTCGGCTGT GGCCACCGCG CGCCGCCCGC GCTGGCTGTG CGCTGGGGCG
     GTGCTTTGGC TGAGCCGACA CCGGTGGCGC GCGGCGGGCG CGACCGACAC GCGACCCCGC

2821 CTGGTGCTGG CGGGTGGCTT CTTTCTCCTC GGCTTCCTCT TCGGTAGGGG GGCGCCTCGC
     GACCACGACC GCCCACCGAA GAAAGAGGAG CCGAAGGAGA AGCCATCCCC CCGCGGAGCG

2881 GGAGCAAACC TCGGAGTCTT CCCCGTGGTG CCGCGGTGCT GGGACTCGCG GGTCAGCTGC
     CCTCGTTTGG AGCCTCAGAA GGGGCACCAC GGCGCCACGA CCCTGAGCGC CCAGTCGACG

2941 CGAGTGGGAT CCTGTTGCTG GTCTTCCCCA GGGGCGGCGA TTAGGGTCGG GGTAATGTGG
     GCTCACCCTA GGACAACGAC CAGAAGGGGT CCCCGCCGCT AATCCCAGCC CCATTACACC

3001 GGTGAGCACC CCTCGAG
     CCACTCGTGG GGAGCTC

FIGURE 32

Potential binding sites on the PSM promoter*
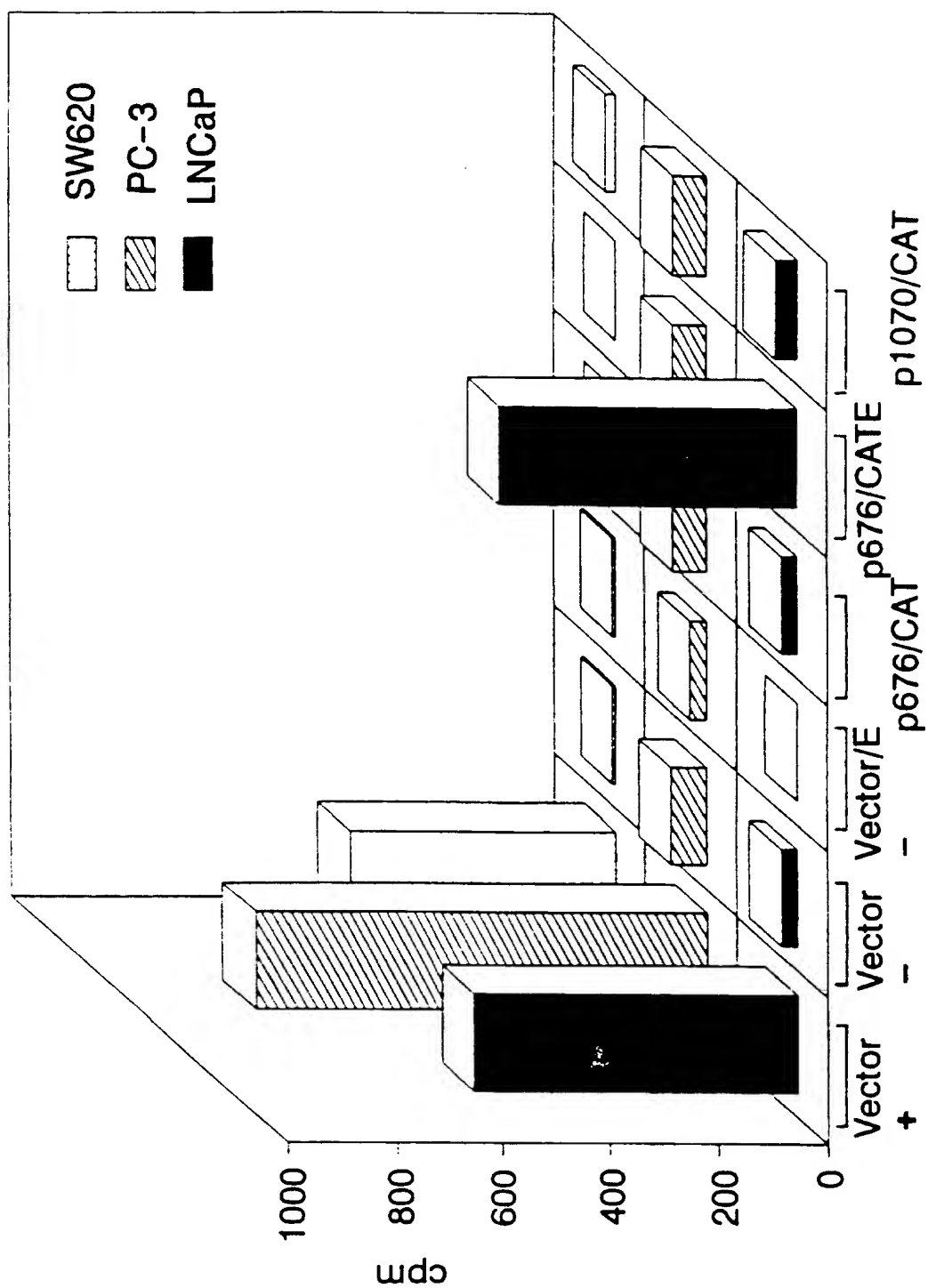
Site	Seq	**Location	#nt matched
AP1	TKAGTCA	1145	7/7
E2-RS	ACCNNNNNNGGT	1940	12/12
		1951	12/12
GHF	NNNTAAATNNN	580	11/11
		753	11/11
		1340	11/11
		1882	11/11
		1930	11/11
		1979	11/11
		2001	11/11
		2334	11/11
		2374	11/11
		2591	11/11
		2620	11/11
		2686	11/11
JVC repeat	GGGNGGRR	1165	8/8
		1175	8/8
		1180	8/8
		1185	8/8
		1192	8/8
NFkB	GGGRHTYYHC	961	10/10
uteroglobi	RYYWSGTG	250	8/8
		921	8/8
		1104	8/8
IFN	AAWAANGAAAGGR	590	13/13	Cell 41.509 (1985)

* the PSM promoter sequence 683XFRVS (Fig 1) starts from the 5' end of the promoter fragment. The 3' region overlapps the previously published PSM cDNA at nt#2485,i.e. the putatative transcription start site is at nt#2485 on sequence 683XFRVS. **The number refered to in this table is in reference to sequence 683XF107 which is the complement and inverse of 683XFRVS.
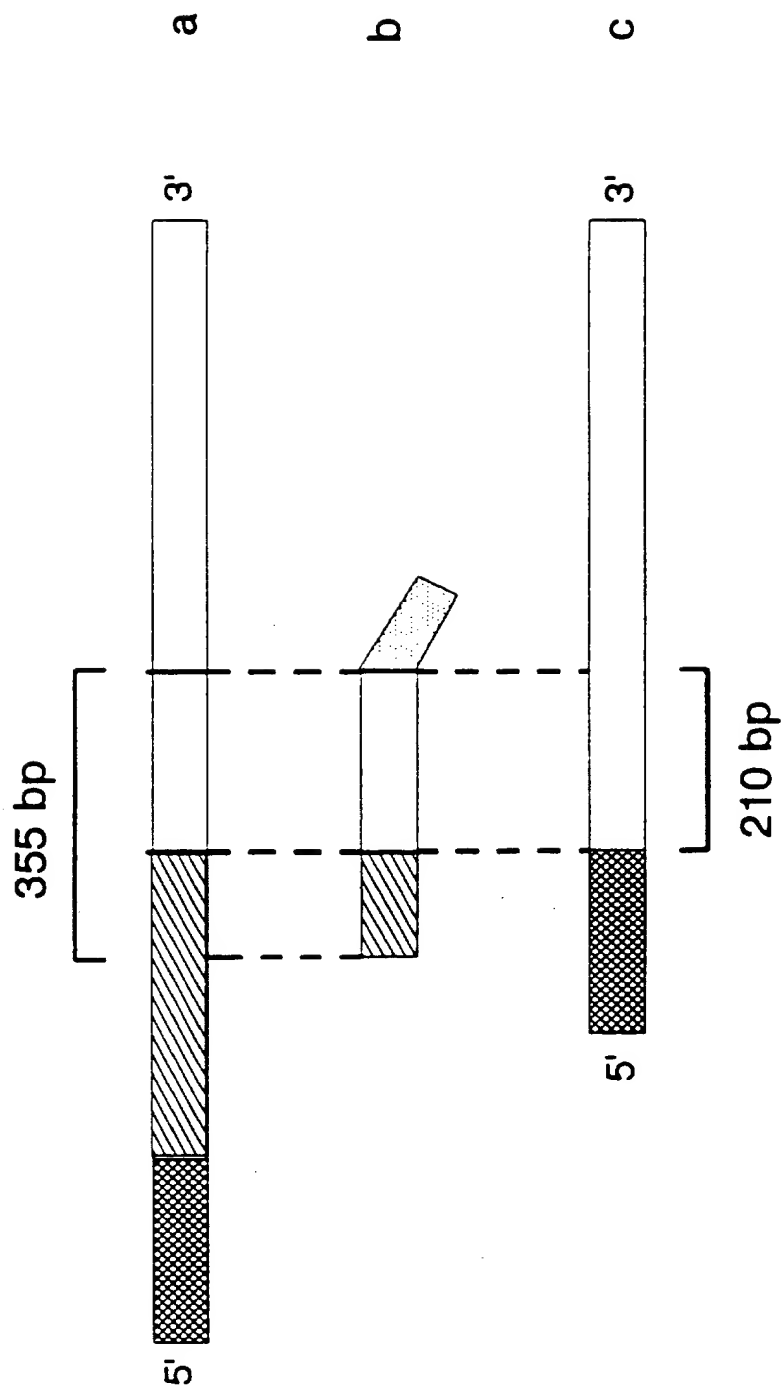
FIGURE 33

FIGURE 34

CTCAAAAGGGGCCGGATTTCCT

TCT TGGAGGCAGATGTTGCCTCTCTCTCTCCTCGGATTGGTTCAGTGCACTCTAGAAACACTGCTGTGGTGGAGAAACT

GGACCCCAGGTCTGGAGCGAATTCCAGCCTGCAGGGCTGATAAGCGAGGCATTAGTGAGATTGAGAGAGACTTTACCC

CGCGGTGGTTGGAGGGCGCAGTAGAGCAGCAGCAGCACAGGCCGCGGGTCCCGGGAGGCCGGCTCTGCTCGCCGCCGAG

ATG TGG AAT CTC CTT CAC GAA ACC GAC TCG GCT GTG GCC ACC GCG CGC CGC CCG CGC TGG CTG
Met Trp Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Ala Arg Arg Pro Arg Trp Leu

TGC GCT GGG GCG CTG GTG CTG GCG GGT GGC TTC TTT CTC CTC GGC TTC CTC TTC GGA TGG TTT
Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe

ATA AAA TCC TCC AAT GAA GCT ACT AAC ATT ACT CCA AAG CAT AAT ATG AAA GCA TTT TTG GAT GAA
Ile Lys Ser Ser Asn Glu Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu

TGG AAA GCT GAG AAC ATC AAG AAG TTC TTA TAT AAT TTT ACA CAG ATA CCA CAT TTA GCA GGA ACA
Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile Pro His Leu Ala Gly Thr

FIGURE 35

FIGURE 36

FIGURE 37

FIGURE 38

FIGURE 39

```
                 10         20         30         40         50         60
                  |          |          |          |          |          |
    1 TTTGCAGACT TGACCAACTT TCTAAGAAAA GCAGAACCAC ACAGGCAAGC TCAGACTCTT
      AAACGTCTGA ACTGGTTGAA AGATTCTTTT CGTCTTGGTG TGTCCGTTCG AGTCTGAGAA


   61 TTATTAAATT CCAGTTTTGA CTTTGCCACT TCTTAGTGGC CTTGAACAAG TTACCGAGTC
      AATAATTTAA GGTCAAAACT GAAACGGTGA AGAATCACCG GAACTTGTTC AATGGCTCAG


  121 CTCTCAGCGT TAGTTACCCT ATTTTAATGA TGAGGATAAT ATTATCTGCC CAAATTATTG
      GAGAGTCGCA ATCAATGGGA TAAAATTACT ACTCCTATTA TAATAGACGG GTTTAATAAC


  181 GTATAGTAAA TATATAGCAT GTAAATCTCC TAGCAGAGTA CTGGGATTTC GCCACTTTAT
      CATATCATTT ATATATCGTA CATTTAGAGG ATCGTCTCAT GACCCTAAAG CGGTGAAATA


  241 TTCTTCTTTA CCAAGATACT CCTATTGGAC TTAATACACA GGACTAGTCT AAGGTATCAC
      AAGAAGAAAT GGTTCTATGA GGATAACCTG AATTATGTGT CCTGATCAGA TTCCATAGTG


  301 CAGGTAGTCC ACTCCTGCTC GGAATCTGAC CCGGGATTAG AGTAGGGCAT GGACCAGATG
      GTCCATCAGG TGAGGACGAG CCTTAGACTG GGCCCTAATC TCATCCCGTA CCTGGTCTAC


  361 GGTTTAAACA AATTCAATAT CTTCCACTAG CTTCACCTTG GGGTTGTAAA AGTTTTTGAA
      CCAAATTTGT TTAAGTTATA GAAGGTGATC GAAGTGGAAC CCCAACATTT TCAAAAACTT


  421 CCACACACTG TGCTCATAAC AATCTTCATC TCTTAAAAGG ATTTTATTCT TCCTGGTATC
      GGTGTGTGAC ACGAGTATTG TTAGAAGTAG AGAATTTTCC TAAAATAAGA AGGACCATAG


  481 CTCACTCTCA TCCCTTGTAT TCCGTGCTCA GTGGCTGACA CAGAAGAGTT CTTTATNNNN
      GAGTGAGAGT AGGGAACATA AGGCACGAGT CACCGACTGT GTCTTCTCAA GAAATANNNN


  541 NNNNNNNNNN CATCCTGTTC ATTTTTCAGA TCTCAGTTCA AGCATCTCGT CCTCAGTGTG
      NNNNNNNNNN GTAGGACAAG TAAAAAGTCT AGAGTCAAGT TCGTAGAGCA GGAGTCACAC


  601 GTGTTNNCTG ATCCCTCACT CTAATCCAAG TCTTTCTGTT TTATGCACAG GTTGGAATCT
      CACAANNGAC TAGGGAGTGA GATTAGGTTC AGAAAGACAA AATACGTGTC CAACCTTAGA


  661 TATTTCCGTT TGCGNNCCAA TCNAATNGTA TTTAATATGC ATGTATATAT GTATGTGCAT
      ATAAAGGCAA ACGCNNGGTT AGNTTANCAT AAATTATACG TACATATATA CATACACGTA


  721 TTGTATGCTA NGCGATTAAG AACTAGAATA ATTAATAATT GGAAGTCTAG AAGTGG
      AACATACGAT NCGCTAATTC TTGATCTTAT TAATTATTAA CCTTCAGATC TTCACC
```

FIGURE 40A

```
            10         20         30         40         50         60
             |          |          |          |          |          |
   1 TGAAAAATAC ATCAAAAATA GGCATGAGAT ACGAGCCTAT AGATAGGACT TATTTTTTAT
     ACTTTTTATG TAGTTTTTAT CCGTACTCTA TGCTCGGATA TCTATCCTGA ATAAAAAATA

  61 TATTGTTGTA TGTATTATTT GTAAAACACA AATTATCAAT ATTACCTCTG ACATTAGGTG
     ATAACAACAT ACATAATAAA CATTTTGTGT TTAATAGTTA TAATGGAGAC TGTAATCCAC

 121 AGATATTCTG AATTTTAATT TCTCTTGCCT ACTTTCACTG AAAAAGAGTC ATGCAAACAG
     TCTATAAGAC TTAAAATTAA AGAGAACGGA TGAAAGTGAC TTTTTCTCAG TACGTTTGTC

 181 ATTTTTAAGT TGCAAACCAA TTGCAAAATA TTTTTTTATC CAACTTCAAT GATAGGTATT
     TAAAAATTCA ACGTTTGGTT AACGTTTTAT AAAAAAATAG GTTGAAGTTA CTATCCATAA

 241 GCTGTTAATT CTAAGATATG CATTAATTGT TTCAACTAAT GGGTGTCAAA CGAGATGTTC
     CGACAATTAA GATTCTATAC GTAATTAACA AAGTTGATTA CCCACAGTTT GCTCTACAAG

 301 TGAAAATGAA GGCAAAAAGG AGATCCACCT TCTACTTTCA TAAAGTTTCT ATCTTCCTCT
     ACTTTTACTT CCGTTTTTCC TCTAGGTGGA AGATGAAAGT ATTTCAAAGA TAGAAGGAGA

 361 GCTGACTCAA ATAAGCATTT AATACATTTT ATAACGAATT AATTATGAAT ATATTTCAAA
     CGACTGAGTT TATTCGTAAA TTATGTAAAA TATTGCTTAA TTAATACTTA TATAAAGTTT

 421 TAAATAAATT ATTTCCAAGT GTTGAAGGAA ATTCAGACTT CTAATTTGCT CTGATTCTGA
     ATTTATTTAA TAAAGGTTCA CAACTTCCTT TAAGTCTGAA GATTAAACGA GACTAAGACT

 481 AACTAAAACA AATGCTCTGT GAGAGTTTGC GTTTCCAGTG AAGTAGCGTG AGAAATCCAA
     TTGATTTTGT TTACGAGACA CTCTCAAACG CAAAGGTCAC TTCATCGCAC TCTTTAGGTT

 541 GTCAGACAGC TACATGAAAC TACATTTACC AGCTCTCTGC CAGACACCAG TGCACGATAG
     CAGTCTGTCG ATGTACTTTG ATGTAAATGG TCGAGAGACG GTCTGTGGTC ACGTGCTATC

 601 CGCAGAACAT GTAGCTAGAT CTCAGTCATA GCTNNNNNNN NNNNNNNNNN AGACCTTGCA
     GCGTCTTGTA CATCGATCTA GAGTCAGTAT CGANNNNNNN NNNNNNNNNN TCTGGAACGT

 661 GTTGGCTTTT AACCTGAAGG AGATAAGGCA AGATTCCAGG GTTTATTTAG AGAAATTACA
     CAACCGAAAA TTGGACTTCC TCTATTCCGT TCTAAGGTCC CAAATAAATC TCTTTAATGT

 721 GGATCTGGGA ATAAAGTAGT TACAAAATTA GTCCCCAACC AGCTTTCATG GAGCTTTCAA
     CCTAGACCCT TATTTCATCA ATGTTTTAAT CAGGGGTTGG TCGAAAGTAC CTCGAAAGTT
```

FIGURE 40B

```
 781 TTATTAATTA TTCTAGTTCT TAATCGCATG CATACAATGC ACATACATAT ATACATGCAT
     AATAATTAAT AAGATCAAGA ATTAGCGTAC GTATGTTACG TGTATGTATA TATGTACGTA


 841 ATTAAAATAC ATGATTGGAC GCAAACGGAA ATAAGATTCC ACCTGTGCAT AAAACAGAAA
     TAATTTTATG TACTAACCTG CGTTTGCCTT TATTCTAAGG TGGACACGTA TTTTGTCTTT


 901 GACTTGGTTA GAGTGAGGGA TCAGGAAACA CCACACTGAG GACGAGATGN NNNNNNNNNN
     CTGAACCAAT CTCACTCCCT AGTCCTTTGT GGTGTGACTC CTGCTCTACN NNNNNNNNNN


 961 NTAGTGGGTG GGGGGCGGAC ATCAATAAAG AACTCTTCTG TGTCAGCCAC TGAGCACGGA
     NATCACCCAC CCCCGCCTG TAGTTATTTC TTGAGAAGAC ACAGTCGGTG ACTCGTGCCT


1021 ATAAAGGGAT GAGAGTGAGG GCAANTACCA GAAGAATAAA ATCCTTTTAA GAGATGAAGA
     TATTTCCCTA CTCTCACTCC CGTTNATGGT CTTCTTATTT TAGGAAAATT CTCTACTTCT


1081 TTGTTATGAG CACAGTGTGT GGNTTCAAAA ATCTTTTAAC AACCCCAAGG TGAAGCTAGT
     AACAATACTC GTGTCACACA CCNAAGTTTT TAGAAAATTG TTGGGGTTCC ACTTCGATCA


1141 TGGAAGATAT TTGAATTTGT TTAAACCCAT CTGGTCCTAG CCCTATTCTT TGAATCCGAA
     ACCTTCTATA AACTTAAACA AATTTGGGTA GACCAGGATC GGGATAAGAA ACTTAGGCTT


1201 GAGGTCAAGA ATTCCGAGCA GAGTGGACTA CCTGTGATAC CTTAGACTAG TCCTGTGTAT
     CTCCAGTTCT TAAGGCTCGT CTCACCTGAT GGACACTATG GAATCTGATC AGGACACATA


1261 TCAAGTCCAA TGAGAGTATC TGTAAGAGAA TAAGTGCGAA ATCCAGATCT
     AGTTCAGGTT ACTCTCATAG ACATTCTCTT ATTCACGCTT TAGGTCTAGA
```

FIGURE 41

```
              10          20          30          40          50          60
              |           |           |           |           |           |
  1 GGATTCTGTT GAGCCCTAGC TCATTATGAT GTCCTGTTGT CCTACCCAAA TAAGACTCAT
    CCTAAGACAA CTCGGGATCG AGTAATACTA CAGGACAACA GGATGGGTTT ATTCTGAGTA


 61 CCCAACTACA TCTCAATAAT TAATGAAGAT GGAAATGAGG TAAAAAATAA ATAAATAAAT
    GGGTTGATGT AGAGTTATTA ATTACTTCTA CCTTTACTCC ATTTTTTATT TATTTATTTA


121 AAAAGAAACA TTCCCCCCCA TTTATTATTT TTTCAAATAC CTTCTATGAA ATAATGTTCT
    TTTTCTTTGT AAGGGGGGGT AAATAATAAA AAAGTTTATG GAAGATACTT TATTACAAGA


181 ATCCCTCTCT AAATATTAAT AGAAATCAAT ATTATTGGAA CTGTGAATAC CTTTAATATC
    TAGGGAGAGA TTTATAATTA TCTTTAGTTA TAATAACCTT GACACTTATG GAAATTATAG


241 TCATTATCCG GTGTCAACTA CTTTCCTATG ATGTTGAGTT ACTGGGTTTA GAAGTCGGGA
    AGTAATAGGC CACAGTTGAT GAAAGGATAC TACAACTCAA TGACCCAAAT CTTCAGCCCT


301 AATAATGCTG TAAANNNNNN AGTTAGTCTA CACACCAATA TCAAATATGA TATACTTGTA
    TTATTACGAC ATTTNNNNNN TCAATCAGAT GTGTGGTTAT AGTTTATACT ATATGAACAT


361 AACCTCCAAG CATAAAAAGA GATACTTTAT AAAAGAGGTT CTTTTTTTCT TTTTTTTTTT
    TTGGAGGTTC GTATTTTTCT CTATGAAATA TTTTCTCCAA GAAAAAAAGA AAAAAAAAAA


421 TCCAGATGGA GTTTTACTCC TGTCAGGCAG GCNGACTGCA GTGGTGCCAT CTCGGCTCAC
    AGGTCTACCT CAAAGTGAGG ACAGTCCGTC CGNCTGACGT CACCACGGTA GAGCCGAGTG


481 TGCAACCTCC ACCTCCCATG TTCAAGGGAT TCTCCTTCCT CAGTCTCCTG AGTAGCTGGG
    ACGTTGGAGG TGGAGGGTAC AAGTTCCCTA AGAGGAAGGA GTCAGAGGAC TCATCGACCC


541 ATTACAGGTG TGCACCACCA CACCCAGCTA ATTTTTGTAT TTTTAATAGA GACAGGGTTT
    TAATGTCCAC ACGTGGTGGT GTGGGTCGAT TAAAAACATA AAAATTATCT CTGTCCCAAA


601 CGATCGATGT TGGCCAGGCT AGTCTCGAAC TCCTGACCTC TAGGTGATCC ACCCGCTCAG
    GCTAGCTACA ACCGGTCCGA TCAGAGCTTG AGGACTGGAG ATCCACTAGG TGGGCGAGTC


661 CTCCCAAAGT TGTAGAATTA CACGTGTGAG GCACTGCGCC TTGCCAGGAG ATACATTTTT
    GAGGGTTTCA ACATCTTAAT GTGCACACTC CGTGACGCGG AACGGTCCTC TATGTAAAAA


721 GATAGGTTTA ATTTATAAAG ACACTGCACA GATTTGAGTT GCTGGGAAAT GCACGGATTC
    CTATCCAAAT TAAATATTTC TGTGACGTGT CTAAACTCAA CGACCCTTTA CGTGCCTAAG


781 CAGTATGCA
    GTCATACGT
```

FIGURE 42


```
               10        20        30        40        50        60
               |         |         |         |         |         |
  1  AATCAAAATA AAACAGTTAA AGTTCATTA CTATAATCAA ACACAAAAAA AATGAATATT
     TTAGTTTTAT TTTGTCAATT TCAAACTAAT GATATTAGTT TGTGTTTTTT TTACTTATAA

 61  ATCTTTTATG TCACTAGAGG CTGAATGAAT CCTTCAGGAT TTGATGATA GTATCAGATA
     TAGAAAATAC AGTCATCTCC GACTTACTTA GGAAGTCCTA AAACTACTAT CATAGTCTAT

121  CCCAGCCACTA TGCTAGAAGT TGTGAAGAAT TCACGAGATG AATAAATCAC AGATTCTGTC
     GGGTCGTGAT ACGATCTTCA ACACTCTTA AGTGCTCTAC TTATTTAGTG TCTAAGACAG

181  CTCAAAATGG TTAGATCTAT TCAGGAAACA AGCTAAAAA ATAACTAAAA
     GAGTTTTACC AATCTAGATA AGTCCTTTGT TTCGATTTTT TATTGATTTT

241  ATCAACCAAA TGAAAAACAA CAATCATAAA ATAAGTAAGT ACCTATAGAA AGAAAGCTC
     TAGTTGGTTT ACTTTTTGTT GTTAGTATTT TATTCATTCA TGGATATCTT TCTTTTCGAG

301  AGAGGAGGTA AAAAGAATCT CCTTAAAAGG AATACTATAT ACTGTAAAAC TGTGACTGAT
     TCTCCTCCAT TTTTCTTAGA GGAATTTTCC TTATGATATA TGACATTTTG ACACTGACTA

361  AGAAGGAA
     TCTTCCTT
```

FIGURE 43A

```
             10         20         30         40         50         60
              |          |          |          |          |          |
  1 TATGGGAAAG TTTTCAGAGG AAATAAGGTA AGGGAAAAGT TATCTCTTTT TTTCTCTCCC
    ATACCCTTTC AAAAGTCTCC TTTATTCCAT TCCCTTTTCA ATAGAGAAAA AAAGAGAGGG

 61 CCAATGTAAA AAGTTATAGT GGGTTTTACA TGTGTAGAAT CATTTTCTTA AAACTTTATG
    GGTTACATTT TTCAATATCA CCCAAAATGT ACACATCTTA GTAAAAGAAT TTTGAAATAC

121 AATACCATTA TTTTCTTGTA TTCTGTGACA TGCCACCTTA CAGAGAGGAC ACATTTACTA
    TTATGGTAAT AAAAGAACAT AAGACACTGT ACGGTGGAAT GTCTCTCCTG TGTAAATGAT

181 GGTTATATCC CGGGGTTAAA TTCGAGCATT GGAATTTGGC CAGTGTAGAT GTTTAGAGTG
    CCAATATAGG GCCCCAATTT AAGCTCGTAA CCTTAAACCG GTCACATCTA CAAATCTCAC

241 AACAGAACAA TTTTTCTGTG CTTACAGTTT ATGGCTGTGG CGTACAAGAA GCATGCACTG
    TTGTCTTGTT AAAAAGACAC GAATGTCAAA TACCGACACC GCATGTTCTT CGTACGTGAC

301 GGTTATTAT TAACTTTCAG TATCTTTGTT TTAAATATTT TCTACAAAAA TGTTTACTAA
    CCAAATAATA ATTGAAAGTC ATAGAAACAA AATTTATAAA AGATGTTTTT ACAAATGATT

361 ATTAAATTGT AGTATGAATT GTTATAAATA ATGAGGTAAA CATTTACACA TAGCAAATTT
    TAATTTAACA TCATACTTAA CAATATTTAT TACTCCTTT GTAAATGTGT ATCGTTTAAA

421 AAAAATTACT GTCATTTGAT TTGTTAATAT ATTTTTCTCT TTAGTGGGAA ATTAAATTAA
    TTTTTAATGA CAGTAAACTA AACAATTATA TAAAAAGAGA AATCACCCTT TAATTTAATT

481 AAAATTCCTT TCGACTGTCA GACAATAGGA TTGCTGTGGT CTACTTGCTT ATTATATTTG
    TTTTAAGGAA AGCTGACAGT CTGTTATCCT AACGACACCA GATGAACGAA TAATATAAAC

541 TAGAGTCTAG AATGCAATCT CACTACACTA TAGACATCTC ANNCTAACGT AGGACAATTC
    ATCTCAGATC TTACGTTAGA GTGATGTGAT ATCTGTAGAG TNNGATTGCA TCCTGTTAAG

601 TGAGAAACTA TTCCAGACCT CCTTATGGGC TTAGCCAAGG NTATCCTTCA GCTGGCATTG
    ACTCTTTGAT AAGGTCTGGA GGAATACCCG AATCGGTTCC NATAGGAAGT CGACCGTAAC

661 CAGGGTGACT TCTNCCTCNN AATCCAGCTC TCTNTCACAG ATGTGATCCA AGAGACACTC
    GTCCCACTGA AGANGGAGNN TTAGGTCGAG AGANAGTGTC TACACTAGGT TCTCTGTGAG

721 ACAATTAATC AACTAGCATT CTAAATTTCA ATTCCAGATC TATTACCTTA ATATGGTAGC
    TGTTAATTAG TTGATCGTAA GATTTAAAGT TAAGGTCTAG ATAATGGAAT TATACCATCG
```

FIGURE 43B

781 TGAAGCTTTN NTCACTGTCA ATTCTGATCA GATATATGAC AATTTTAAAT TATTTGCAGT
    ACTTCGAAAN NAGTGACAGT TAAGACTAGT CTATATACTG TTAAAATTTA ATAAACGTCA

841 GTGTAAGAAA CGCTTCAGGT AGTTTAAATT TAAGGCT
    CACATTCTTT GCGAAGTCCA TCAAATTTAA ATTCCGA

FIGURE 44A

```
              10         20         30         40         50         60
               |          |          |          |          |          |
   1 CTCCTTTGGC CCCTGCCAGC TGGGCATTTT TAACCTAGTT TACACAGTGT CTTTTTTTCC
     GAGGAAACCG GGGACGGTCG ACCCGTAAAA ATTGGATCAA ATGTGTCACA GAAAAAAAGG

  61 TTATTTTAAA TTGGTTGTTC CAGATTCGGT AATATCAATT TTTAATATTA CACTTAAATG
     AATAAAATTT AACCAACAAG GTCTAAGCCA TTATAGTTAA AAATTATAAT GTGAATTTAC

 121 AGTACCAGAA CTTTATCTTC AACCTTTTTC TCATTAGGCC TACAACATAG GACATCTCGG
     TCATGGTCTT GAAATAGAAG TTGGAAAAAG AGTAATCCGG ATGTTGTATC CTGTAGAGCC

 181 ATAGAATTTC CTTTTCTTTT TGCTACTATA AGCTGCTAAA ATCCTCAGAA CATCAGATTT
     TATCTTAAAG GAAAAGAAAA ACGATGATAT TCGACGATTT TAGGAGTCTT GTAGTCTAAA

 241 AGAAATGTTC TTATTAGTGG TAGTGAGCAT TTGCTATTTC CTACCACTAG CTTACAAATA
     TCTTTACAAG AATAATCACC ATCACTCGTA AACGATAAAG GATGGTGATC GAATGTTTAT

 301 TAATAAGCAA GTAGACCCCA CAGGCCAAAT TCCTATTTGT TCTACAGTCG AAAGGGAATT
     ATTATTCGTT CATCTGGGGT GTCCGGTTTA AGGATAAACA AGATGTCAGC TTTCCCTTAA

 361 TTTTAAAATT TAATTTCCAC TAAAGAGAAA AATATATTAA CAATCAAATT GACAGTCGAT
     AAAATTTTAA ATTAAAGGTG ATTTCTCTTT TTATATAATT GTTAGTTTAA CTGTCAGCTA

 421 TTTAATTGCT ATGTGTAATT GTTTTCCCTC ATTATTTATA ACAATTCATA CTACAATTTA
     AAATTAACGA TACACATTAA CAAAAGGGAG TAATAAATAT TGTTAAGTAT GATGTTAAAT

 481 ATTTAGTAAA CATTTTTGTA GACCATATTT AAAACAAAGA TACTGAAAGT TAATATAAAC
     TAAATCATTT GTAAAAACAT CTGGTATAAA TTTTGTTTCT ATGACTTCA ATTATATTTG

 541 TTAGTGCATG CTCTCTGTAG GCCACAGCCA TAACCTGTAA GCACAGAAAA ATTTGTTCTG
     GGTCACGTAC GAGAGACATC CGGTGTCGGT ATTGGACATT CGTGTCTTTT TAAACAAGAC

 601 TTACTCTAAA CATCTATACT GGCCAAATTC CAATGCTCGA ATTTAACCCC GGGATATAAC
     AATGAGATTT GTAGATATGA CCGGTTTAAG GTTACGAGCT TAAATTGGGG CCCTATATTG

 661 TTAGTAAATG TGTCCTCTCT GTCAAGGTGG GCATGTCACA GAATACAGAA CAATCAATGG
     GATCATTTAC ACAGGAGAGA CAGTTCCACC CGTACAGTGT CTTATGTCTT GTTAGTTACC

 721 TATTCATAAA GTTTTAAGAA AATGATTCTA CACATGTAAA ACCCACTATA ACTTTTTACA
     ATAAGTATTT CAAAATTCTT TTACTAAGAT GTGTACATTT TGGGTGATAT TGAAAAATGT
```

FIGURE 44B

781 TTGGGGGAGA GAAAAAAAGA GATAATTTTT ACCTTACCTT ATTTCCTCTG AAAACTTTCC
     AACCCCCTCT CTTTTTTTCT CTATTAAAAA TGGAATGGAA TAAAGGAGAC TTTTGAAAGG

841 CATATCTGGC AATTACAATT TTCCCAGAGG AATTGATTTT CATGTCCCGT TCC
     GTATAGACCG TTAATGTTAA AAGGGTCTCC TTAACTAAAA GTACAGGGCA AGG

FIGURE 45A

```
            10         20         30         40         50         6:
            |          |          |          |          |          |
   1 GATGCTATTT GGGCAATTTC TTATTGACAG TTTTGAAATG TTAGGCTTTT ATCTCCATTT
     CTACGATAAA CCCGTTAAAG AATAACTGTC AAAACTTTAC AATCCGAAAA TAGAGGTAAA

  61 TTTAGTACTT AAATTTTCCA ACATGGGTGT TGCTTGTTAT TTTATCAGTA TAAAATAGAA
     AAATCATGAA TTTAAAAGGT TGTACCCACA ACGAACAATA AAATAGTCAT ATTTTATCTT

 121 GAGTGGTTCT GTTCTGGAAT TTAGTATATA CATGAGTATC TAGTGTATGT CAGCCATGAA
     CTCACCAAGA CAAGACCTTA AATCATATAT GTACTCATAG ATCACATACA GTCGGTACTT

 181 AATGAACCTT TCAGATGTTT AACTTCAGGG AACCTAATTG AGTCATTGCT CCAGACATTG
     TTACTTGGAA AGTCTACAAA TTGAAGTCCC TTGGATTAAC TCAGTAACGA GGTCTGTAAC

 241 TTGCTTTGAA CCCACTATAT TNNNNNNNCT CGGGCAATCC CTCAGTGTGG CAAGGATACT
     AACGAAACTT GGGTGATATA ANNNNNNNGA GCCCGTTACT GAGTCACACC GTTCCTATGA

 301 ACTGCAGGCC TGTTTCTGGA AGGCACTGGA CTCCTCTGAT GCAAACTTTG GCCAGGGACT
     TGACGTCCGG ACAAAGACCT TCCGTGACCT GAGGAGACTA CGTTTGAAAC CGGTCCCTGA

 361 CCTTGATAGC TCTTAAATAG ATGCTGCACC AACACTCTCT TTCTTTTCTC TCTTTTTCTT
     GGAACTATCG AGAATTTATC TACGACGTGG TTGTGAGAGA AAGAAAAGAG AGAAAAAGAA

 421 TATTCAATAT TAGACTACAA GCATTTTAAT GATTCTCAG GGTTTCTAGC TCTCTCTCAT
     ATAAGTTATA ATCTGATGTT CGTCAGATTC CTAAGAGTC CCAAAGATCG AGAGAGAGTA

 481 TTCACACATG CTTTCCTAGT AATCTCTACT CATATATCTT ACTGCTACGC TGGGGCCAGA
     AAGTGTGTAC GAAAGGATCA TTAGAGATGA GTATATAGAA TGACGATGCG ACCCCGGTCT

 541 TAACNNNNNN CTTCCATTTT GTTTTATCT CTATTCTTCT TCCCCTTCTG CTTTCATTAT
     ATTGNNNNNN GAAGGTAAAA CAAAAATAGA GATAAGAAGA AGGGGAAGAC GAAAGTAATA

 601 TGAAACTTTC TGCTTTCATT ATTGAAACTT TCCCAGATTT GTTCTGCTTA ACCTGGCATT
     ACTTTGAAAG ACGAAAGTAA TAACTTTGAA AGGGTCTAAA CAAGACGAAT TGGACCGTAA

 661 GGAACTGTTT CCTCTTCCCT GTGCTGCTTT CTCCCATTGC CATGTCCTTT TTTTTTTTTT
     CCTTGACAAA GGAGAAGGGA CACGACGAAA GAGGGTAACG GTACAGGAAA AAAAAAAAAA

 721 TTTTTTTTTT TGAGACAGTG TCACTCTGTT GCCCAGGCTG GAGTGCAATG GTGCAATCTT
     AAAAAAAAAA ACTCTGTCAC AGTGAGACAA CGGGTCCGAC CTCACGTTAC CACGTTAGAA
```

FIGURE 45B

```
781  GGCCACTGCA ACCCCGACTC CGCGTTCAAG TGATTCTCTA CCTGCCTCAG CCTCCTGAGT
     CCGGTGACGT TGGGGCTGAG GCCCAAGTTC ACTAAGAGAT GGACGGAGTC GGAGGACTCA

841  AGCTGGGATT ACAGGTGCCA CCACTATGCC GGCTGATTTT GTATTTTAGT AGAGATGGGT
     TCGACCCTAA TGTCCACGGT GGTGATACGG CCGACTAAAA CATAAAATCA TCTCTACCCA

901  TCACATGCAG ATCAGCTGTT CCGACTCTGA CCAGNNNNNN NNNNNNNNNN ATCAAAGTCA
     AGTGTACGTC TAGTCGACAA GGCTGAGACT GGTCNNNNNN NNNNNNNNNN TAGTTTCAGT

961  GCCAAAGTGC TAGGCTTAGA GTAATTGTGT AATTTCCACA CAAGTGCAAC CTAGTGTAAT
     CGGTTTCACG ATCCGAATCT CATTAACACA TTAAAGGTGT GTTCACGTTG GATCACATTA

1021 GCCTCAAGAA TGTNNNTATG AATGTCTCGA ACGTTAGTAA CTAATAACAA GTAGTTAGTT
     CGGAGTTCTT ACANNNATAC TTACAGAGCT TGCAATCATT GATTATTGTT CATCAATCAA

1081 TATAGATGTA TCCTAGTATG TAGCA
     ATATCTACAT AGGATCATAC ATCGT
```

FIGURE 46A

```
            10        20        30        40        50        60
            |         |         |         |         |         |
  1 CACAAAAAAA GATTATTAGC CACAAAAAAA CCTTGAAGTA ACGCATTAAA ATGTTAATGG
    GTGTTTTTTT CTAATAATCG GTGTTTTTTT GGAACTTCAT TGCGTAATTT TACAATTACC


 61 ATTCACTTTA TTGAGCATCT GCTCATAATA CTTTAATGAG TGCAAAGTGC TTTGAATATA
    TAAGTGAAAT AACTCGTAGA CGAGTATTAT GAAATTACTC ACGTTTCACG AAACTTATAT


121 ATACGTCATT TAAACCTTAC CATAATTCTG AGGAATTGCT ACCTCCACTT CACAGATGGG
    TATGCAGTAA ATTTGGAATG GTATTAAGAC TCCTTAACGA TGGAGGTGAA GTGTCTACCC


181 GCACAGGAGG CTTAGATAAC ATGCCCAAAG TCATGCTTCT AGTAAATGGA TATAATTAAG
    CGTGTCCTCC GAATCTATTG TACGGGTTTC AGTACGAAGA TCATTTACCT ATATTAATTC


241 ATTCAAATTA TTGATAAGAA TTTGATCTGC CTTACCAGTA TCTAGTAGTA AATCTAAAG
    TAAGTTTAAT AACTATTCTT AAACTAGACG GAATGGTCAT AGATCATCAT TTAGATTTTC


301 CGCTTTCCAG AGCATGTGCT GTTGATAGAG CTTGATGTCT AACTCTCTGA AATTTTCCAT
    GCGAAAGGTC TCGTACACGA CAACTATCTC GAACTACAGA TTGAGAGACT TTAAAAGGTA


361 TCTTATTTGT CTCACTGGTA TATAGTTATT TTTACTACT TTCATACACC TACTAAGAAG
    AGAATAAACA GAGTGACCAT ATATCAATAA AAAATGATGA AAGTATGTGG ATGATTCTTC


421 ACAGGAGGAT CAAAGATAGG ATTTCATTTA GAATGCCTAA AGCTTCACGT ATTTTAATTC
    TGTCCTCCTA GTTTCTATCC TAAAGTAAAT CTTACGGATT TCGAAGTGCA TAAAATTAAG


481 AGAATAAGAT TCAGGCAGAC CACCAGTATA TCCCATGGTC CCTGGTTATC TTTCAGCAGG
    TCTTATTCTA AGTCCGTCTG GTGGTCATAT AGGGTACCAG GGACCAATAG AAAGTCGTCC


541 TGACCGAGAA AGAAAACATG GTAATGTTTA TGAAATGGTG GGTTCTTGTA GTTTCACTTC
    ACTGGCTCTT TCTTTTGTAC CATTACAAAT ACTTTACCAC CCAAGAACAT CAAAGTGAAG


601 AACATATCTG CCTTTACTGT ATTAAGATGA TGGATTAACT TATTCTTGAT ATGGGCATGT
    TTGTATAGAC GGAAATGACA TAATTCTACT ACCTAATTGA ATAAGAACTA TACCCGTACA


661 AAAACAATAT ACTTTTACTA AACAGCTACA GAGAGACAAA TGTGTTTCCA GACAAACTTA
    TTTTGTTATA TGAAAATGAT TTGTCGATGT CTCTCTGTTT ACACAAAGGT CTGTTTGAAT


721 AGAGACTGAG TGTTCAAACT GAATAATCTC GACCTTAATT GTAACTATAT TTTATGAAAT
    TCTCTGACTC ACAAGTTTGA CTTATTAGAG CTGGAATTAA CATTGATATA AAATACTTTA
```

FIGURE 46B

781 CCAGCTGTAA GGCAAAACAG ACTCTTGGCT ACACGGCATT TGTCTGTTAA TGATACTCAA
    GGTCGACATT CCGTTTTGTC TGAGAACCGA TGTGCCGTAA ACAGACAATT ACTATGAGTT

641 CCTTAACCGT CACTTAATAA TGCTGAATAA TGTCATTAAT CTGAGATGTT AGTATGATCA
    GGAATTGGCA GTGAATTATT ACGACTTATT ACAGTAATTA GACTCTACAA TCATACTAGT

901 ATGGGAATCA CTGCTGAGCT CTCGAAGCCC
    TACCCTTAGT GACGACTCGA GAGCTTCGGG

FIGURE 47A

```
                                                          261 CTCAAAAGGGGCCGGAATTCCTT
CTCCTCGAGCCAGATGTTGCCTCTCTCTCCTCCTCGGATTGTTCAGTTACTCTATAAACACTCACTGTGGTGAAAAACTGGACCCCAGGTCTGAGGCGAATTCAACCTGCAGGGCT   -239
GATAAGCGAGGCATTAGTGAGATTGAGAGAGACTTTACCCCACGTGATTGTTGAAAACCGGGCCAGTAGAGCACAACACCACACGGGCGGTCCCCCGGAGAGCCGGCTCTCGCTCGCGGGGAG  -120
                                                                                                                          -1

ATG TGG AAT CTC CTT CAC GAA ACC GAC TCG GCT GTG GCC CGC   CTG TGG CGT GGG GCG CTG GTG GCG GGT       90
Met Trp Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Arg   Leu Trp Arg Gly Ala Leu Val Ala Gly       30

GCC TTC TTT CTC CTC GGC TTC CTC GGG TGG        Intron                                                180
Gly Phe Phe Leu Leu Gly Phe Leu Gly Trp        TTT ATA TCC AAT GAA GCT ACT AAC ATT ACT                60
                                               Phe Ile Ser Asn Glu Ala Thr Asn Ile Thr

TTT TTG GAT GAA TTG AAA GCT GAG AAC ATC AAG AAG   TTC TTA TAT AAT TTT ACA CCA CAT ATA CCA AAA GCA      270
Phe Leu Asp Glu Leu Lys Ala Glu Asn Ile Lys Lys   Phe Leu Tyr Asn Phe Thr Pro His Ile Pro Lys Ala      90

CAG CTT GCA AAG CAA ATT CAA TCC CAG TGG AAA GAA TTT GGC   GAT TCT GTT GAG CTA GCA CAT TAT GAT GTC      360
Gln Leu Ala Lys Gln Ile Gln Ser Gln Trp Lys Glu Phe Gly   Asp Ser Val Glu Leu Ala His Tyr Asp Val     120

AAT AAG ACT CAT CCC AAC TAC ATC TCA ATA ATT GAA GAT GGA      Intron
Asn Lys Thr His Pro Asn Tyr Ile Ser Ile Ile Glu Asp Gly      AAT GAG ATT TTC AAC ACA TTT TCA          450
                                                             Asn Glu Ile Phe Asn Thr Phe Ser          150

TAT GAA AAT GTT TCG GAT ATT GTA CCA CCT TTC TCT CCT CAA   GGC TTC TCT CCT CAA GAG GGC GAT CTA         540
Tyr Glu Asn Val Ser Asp Ile Val Pro Pro Phe Ser Pro Gln   Gly Met Pro Ser Ala Phe Val Tyr Ala        180
```

FIGURE 47B

```
CGA ACT GAA GAC TTC TTT AAA TTG GAA CGA GAC ATG AAA ATC AAT TAC TCT GAG AAA ATT GTA ATT GCC AGA TAT GGG AAA GTT TTC AGA   630
Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met Lys Ile Asn Tyr Ser Glu Lys Ile Val Ile Ala Arg Tyr Gly Lys Val Phe Arg   210
                              Intron

GGA AAT AAG GTT GTT AAT CAG CTG CTG CAA AAT GGA GCC TCC GAC CCT GCT TTT TAC GTG CCT GTG   720
Gly Asn Lys Val Val Asn Gln Leu Gly Ala Ala Tyr Gly Ala Ser Asp Pro Ala Phe Tyr Val Pro Gly Lys   240

TCC TAT CCA GAT GGT GGT AAT CCT GTC CAG CGT GTC ATC AAT CTA CTG GTA GGA GAC CCT CTC ACA CCA   810
Ser Tyr Pro Asp Gly Gly Asn Pro Val Gln Arg Val Ile Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro   270

GGT TAC CCA AAT GAA TAT CGT GGT TGT GTT CAT CCT CCA ATT GGA TAT   900
Gly Tyr Pro Asn Glu Tyr Arg Gly Ala Val Ala Val His Pro Ile Gly Tyr   300
                              Intron

GAT GCA CAG AAG CTC CTA GAA GAG ATG ATG GGC TCA GCA CCA GAT AGT ATT CCA AAT GTG CCC TAC AAT GTT GGA   990
Asp Ala Gln Lys Leu Leu Glu Glu Met Met Gly Ser Ala Pro Asp Ser Arg Ile Pro Asn Val Pro Tyr Asn Val Gly   330

CCT GGC TTT ACT GGA AAC TTT TCT ACA GGA GTG AAA AAT GTC AAG ATC CAC ATC CAC AGA ATT TAC AAT GTG ATA GGT   1080
Pro Gly Phe Thr Gly Asn Phe Ser Thr Gly Val Lys Asn Val Lys Met His Ile His Ser Thr Arg Ile Tyr Asn Val Ile Gly   360
```

FIGURE 47C

```
ACT CTC AGA GGA GTO GAA CCA GAC AGA TAT GTC ATT CTG GGA GCT CAC CGG GAC TCA TGG GTG TTT GGT ATT GAC CCT CAG AGT 1170
Thr Leu Arg Gly Ala Val Glu Pro Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly Ile Asp Pro Gln Ser  390

GGA GCA GCT GTT GTT CAT GAA ATT GTG AGC AGG AAC TTT GGA ACA TGG AGA CCT AGA AGA ATT TTG TTT GCA ACC TTT GCA AGC 1260
Gly Ala Ala Val Val His Glu Ile Val Ser Arg Asn Phe Gly Thr Trp Arg Pro Arg Arg Ile Leu Phe Ala Thr Phe Ala Ser  420

TGG GAT GCA CAA GAA TTT GGT CTT CTT GGT TCT GAG GTA GAG GAG AAT TCA AGA CTC CTT CAA GAG CTT GGC GTO GCT TAT ATT 1350
Trp Asp Ala Gln Glu Phe Gly Leu Leu Gly Ser Glu Val Glu Glu Asn Ser Arg Leu Leu Gln Glu Leu Gly Val Ala Tyr Ile  450

AAT GCT GAC ATA GAA TCT ATA GGA GAC AAC TAC ACT TAC GTA CAC TTG ATG TAC AAC CTA ACA AAA GAG CTA ACA AAA GAG 1440
Asn Ala Asp Ile Glu Ser Ile Gly Asp Asn Tyr Thr Tyr Val His Leu Met Tyr Asn Leu Thr Lys Glu Leu Thr Lys Glu  480

CTO AAA CCT GAT GAA TTT GGA TCT ACT TCT ATG AAA AAA AGT CCT ATC GGA AGT GGC ATG CCC 1530
Leu Lys Pro Asp Glu Phe Gly Ser Thr Ser Met Lys Lys Ser Pro Ile Gly Ser Gly Met Pro  510

AOG ATA AOC TTG GGA TCT GTG AAT GAA GTC CAA CCA GGA ATT TCA GGC AGA GCA OOG TAT ACT AAA AAT 1620
Arg Ile Ser Leu Gly Ser Val Asn Glu Val Gln Pro Gly Ile Ser Gly Arg Ala Ala Tyr Thr Lys Asn  540

TGG GAA ACA AAC TTC AGC GGC TAT CAC CTG TAT GTC ATT TCA GAG AAG TTT TAT GAT CCA ATG TTT 1710
Trp Glu Thr Asn Phe Ser Gly Tyr His Leu Tyr Val Ile Ser Glu Lys Phe Tyr Asp Pro Met Phe  570
```

FIGURE 47D

```
AAA TAT CAC CTC ACT GTG GCC CAG GTT CGA GGA ATG GTG TTT GAG CTA GTG AAT TCC ATA GTG CTC CCT TTT GAT TGT CGA GAT TAT  1800
Lys Tyr His Leu Thr Val Ala Gln Val Arg Gly Met Val Phe Val Leu Val Asn Ser Ile Val Leu Pro Phe Asp Cys Arg Asp Tyr   600

GCT GTA GTT TTA GTT TAT GCT AAG AAA ATC TAC AGT ATT TTT ATG AAA CAT CCA CAG GAA ATG ACA AAG GTA TCA TTT GAT  1890
Ala Val Val Leu Val Tyr Ala Lys Lys Ile Tyr Ser Ile Phe Met Lys His Pro Gln Glu Met Thr Lys Val Ser Val Phe Asp   630

TCA CTT TTT TCT GCA GTA AAG AAT TTT ACA GAA ATT GTT TTC AAG TTC AGT GAG AGA ATT CCA AGC AAA AGC AAC CCA ATA GTA  1980
Ser Leu Phe Ser Ala Val Lys Asn Phe Thr Glu Ile Val Phe Lys Phe Ser Glu Arg Ile Pro Ser Lys Ser Asn Pro Ile Val   660

TTA AGA ATG ATG AAT GAT CAA CTC ATG TTC CTG GAA AGA GCA ATT ATT GAT CCA TTA GAG CGG TTA CCA GAC AGG CCT TAT TTT TAT ATT AGG CAT GTC  2070
Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu Arg Ala Ile Ile Asp Pro Leu Gly Arg Pro Asp Arg Pro Tyr Phe Tyr Ile Arg His Val   690

ATC TAT GCT CCA AGC AGC CAC AAG TAT GCA AGT AAG AAC TTG TTC CCA GGA TTC GGA AGC ACC ATT TAT GCT CTG TTT GAT ATT GAA AGC AAA GTG GAC  2160
Ile Tyr Ala Pro Ser Ser His Lys Tyr Ala Ser Lys Asn Leu Phe Pro Gly Phe Gly Ser Thr Ile Tyr Ala Leu Phe Asp Ile Glu Ser Lys Val Asp   720

CCT TCC AAG GCC TGG GGA AAG GTG AAG AGA CAG ATT TAT GTT GTT GCA GCC TTC ACA GTG CAG GCA GAG ACT TTG AGT GAA GTA GCC  2250
Pro Ser Lys Ala Trp Gly Lys Val Lys Arg Gln Ile Tyr Val Val Ala Ala Phe Thr Val Gln Ala Glu Thr Leu Ser Glu Val Ala   750

TAA GAGGGATTCTTAGAGAGAATCGTATTGAATTGTGTGTATGTCACTCAAAGAATCGTAATTGGTATATTGAATTTAAAATTTAAAATAAGTTGAATATTA  2368
---

TATATAAAAAAAAAAAAAAAAAAAAA  2393
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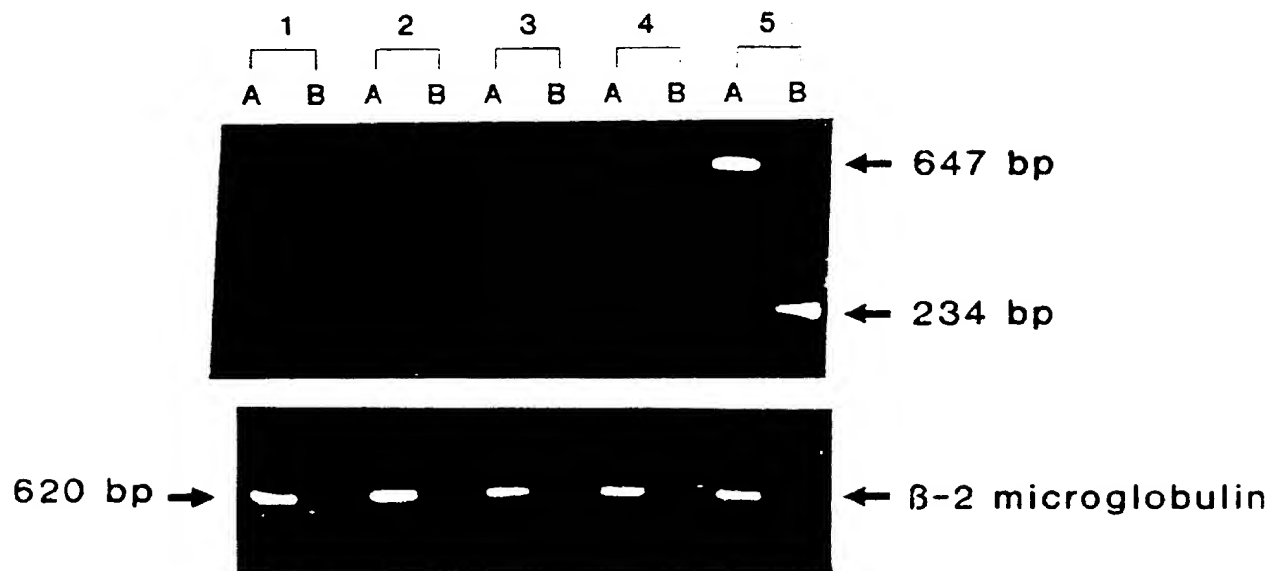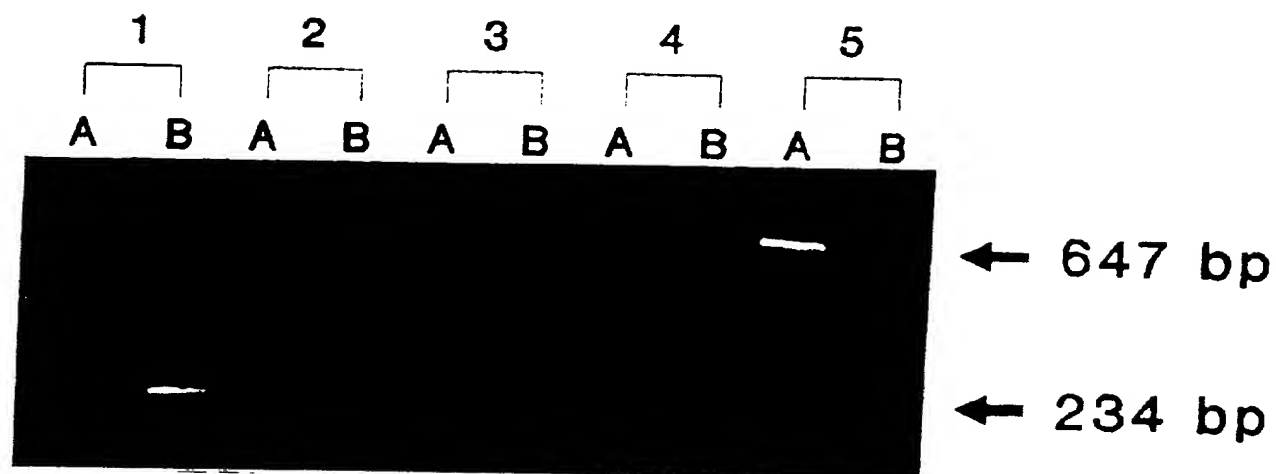
FIGURE 48
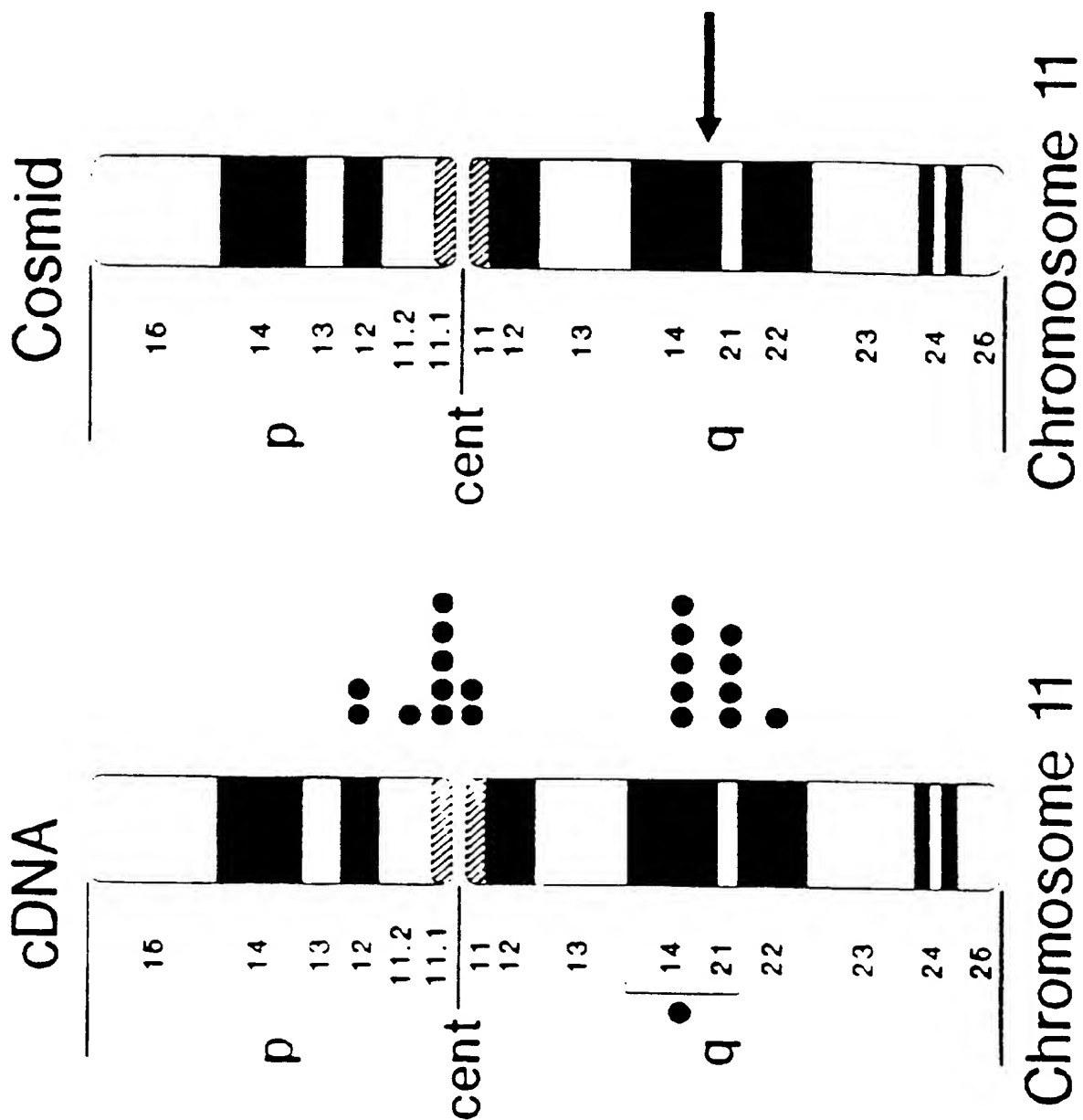
FIGURE 49

FIGURE 50

FIGURE 51

♂ ♀ M H 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
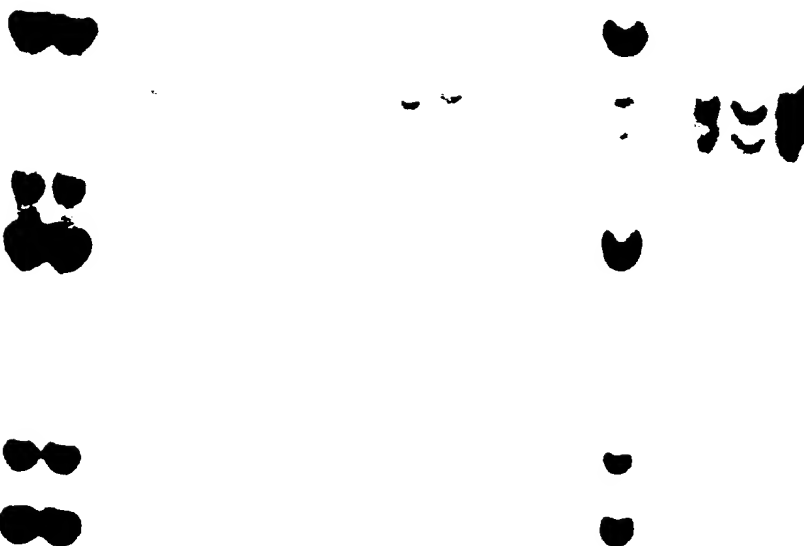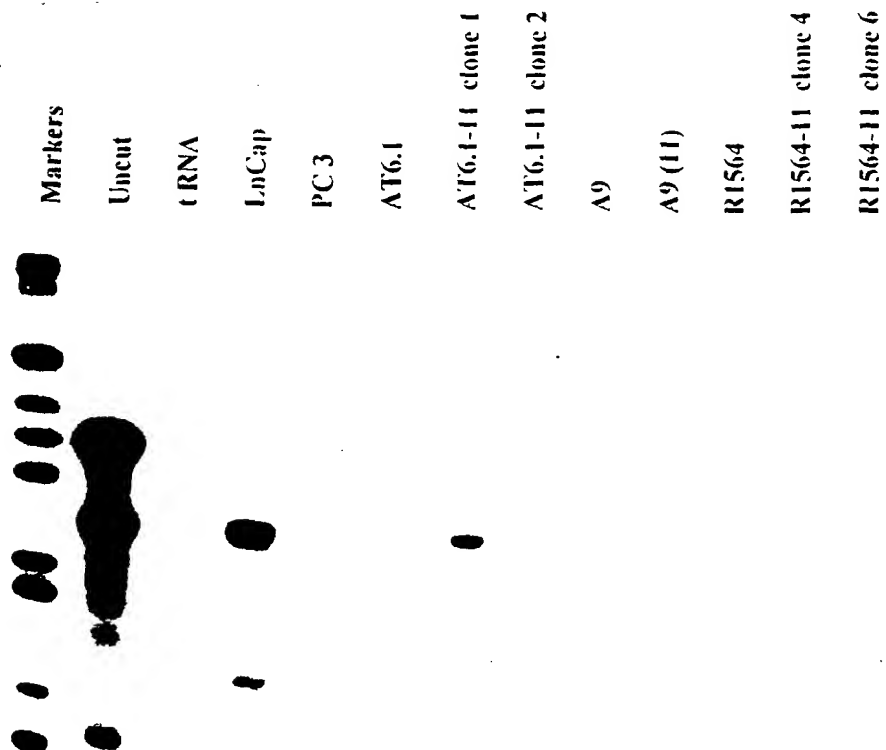
FIGURE 52

FIGURE 53

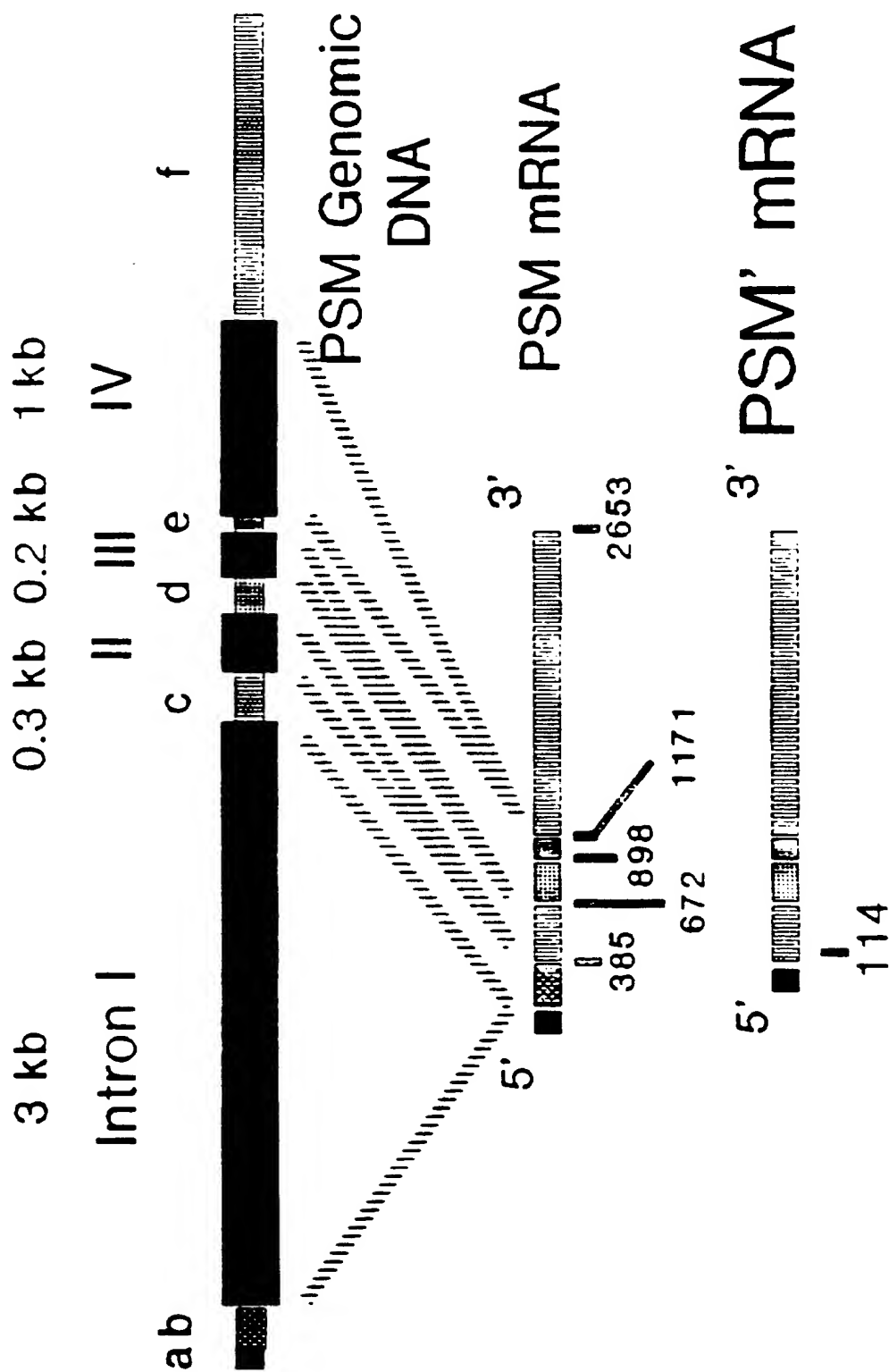| TISSUE/CELL LINE	CANCER CELL TYPE	PSM DNA	PSM RNA
HUMAN PROSTATE	NA	+	+
HUMAN MAMMARY	NA	+	–
AT6.1	RAT PROSTATIC ADENOCARCINOMA	–	–
AT6.1-H-CL1	"	+	–
AT6.1-H-CL2	"	–	–
R1S64	RAT MAMMARY ADENOCARCINOMA	–	–
R1S64-H-CL2	"	+	–
R1S64-H-CL4	"	+	–
R1S64-H-CL5	"	+	–
R1S64-H-CL6	"	+	–
A9	MOUSE FIBROSARCOMA	–	–
A9(H)	"	+	–
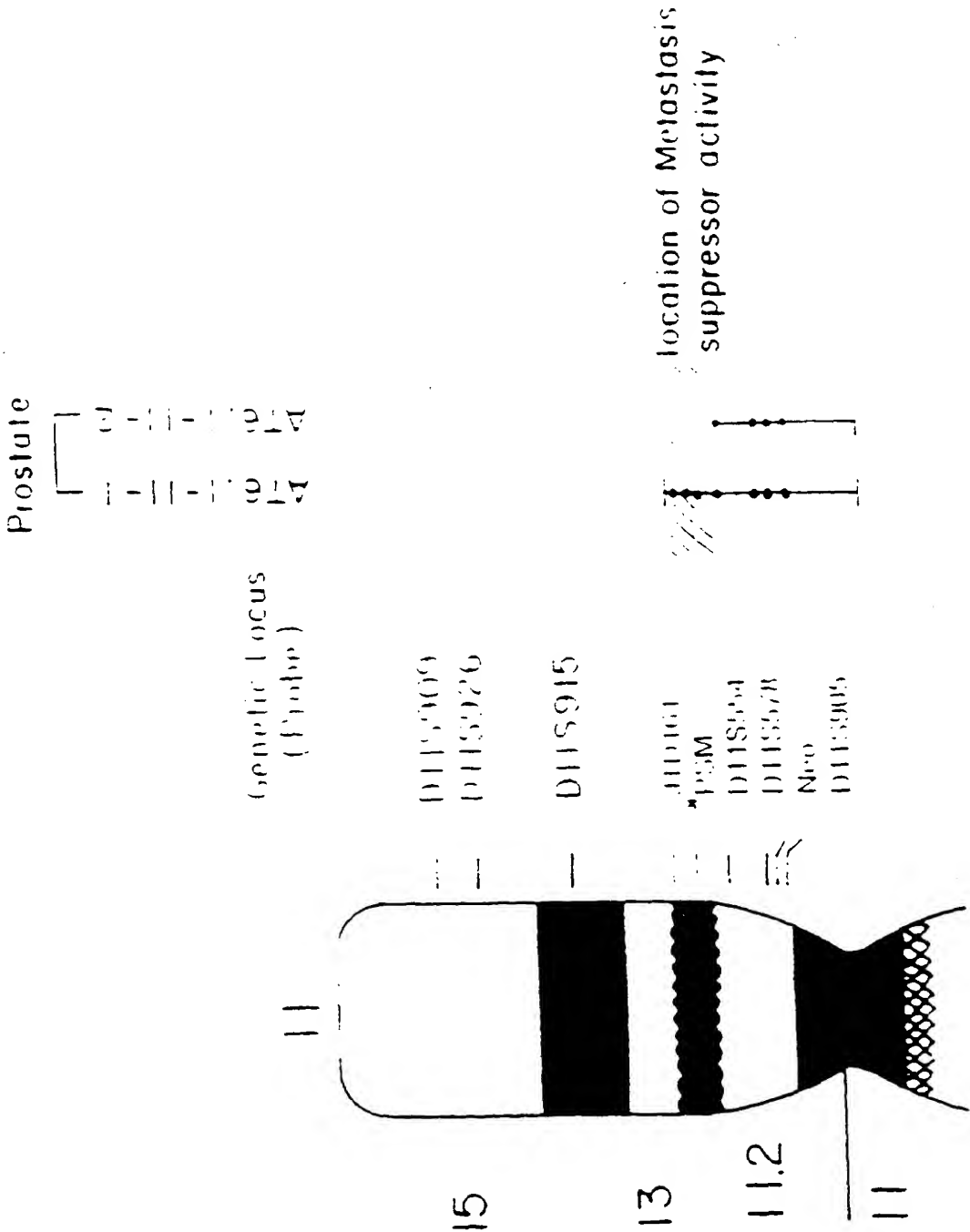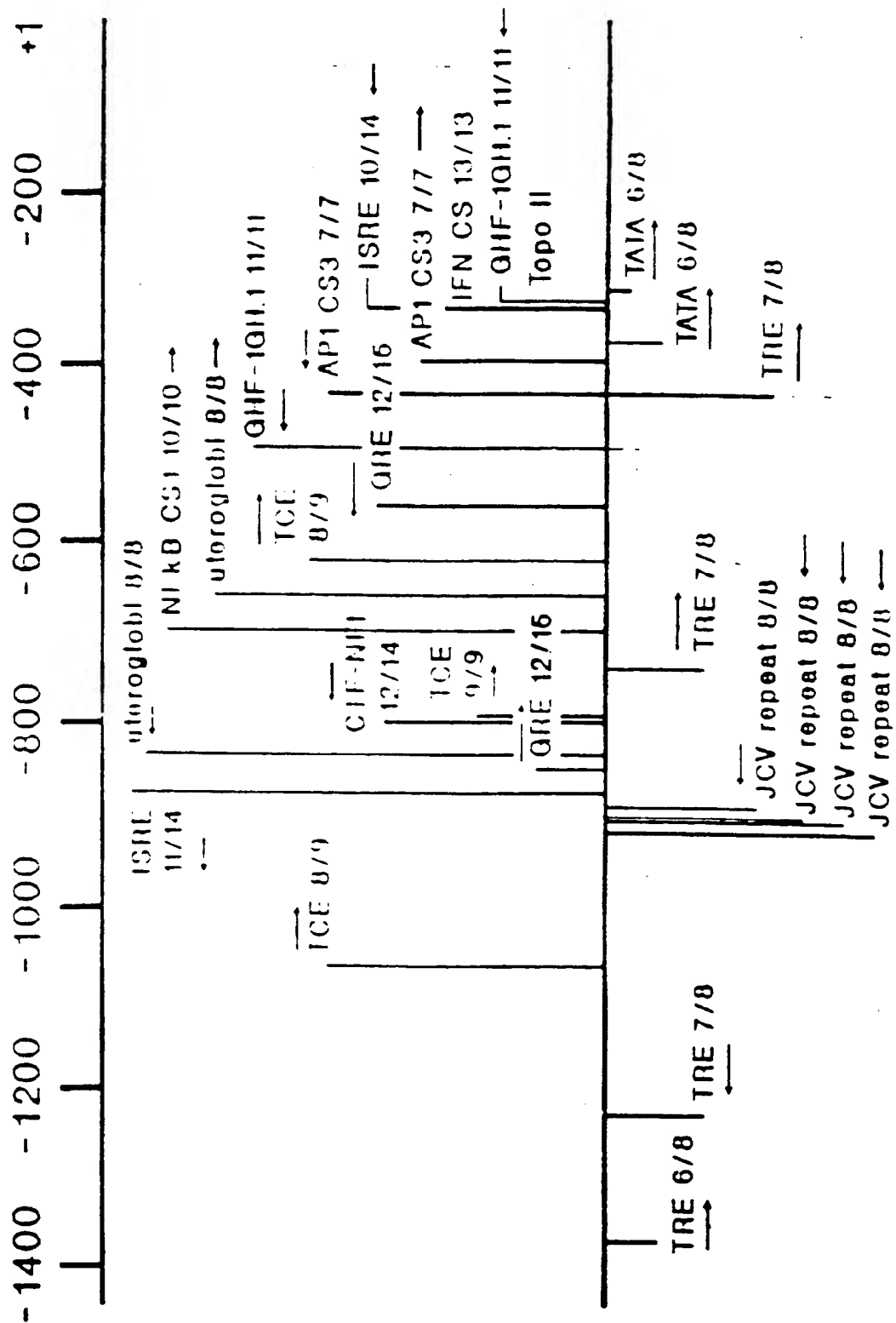
FIGURE 54

FIGURE 55

FIGURE 56

Genomic Organization of PSM Gene

FIGURE 57

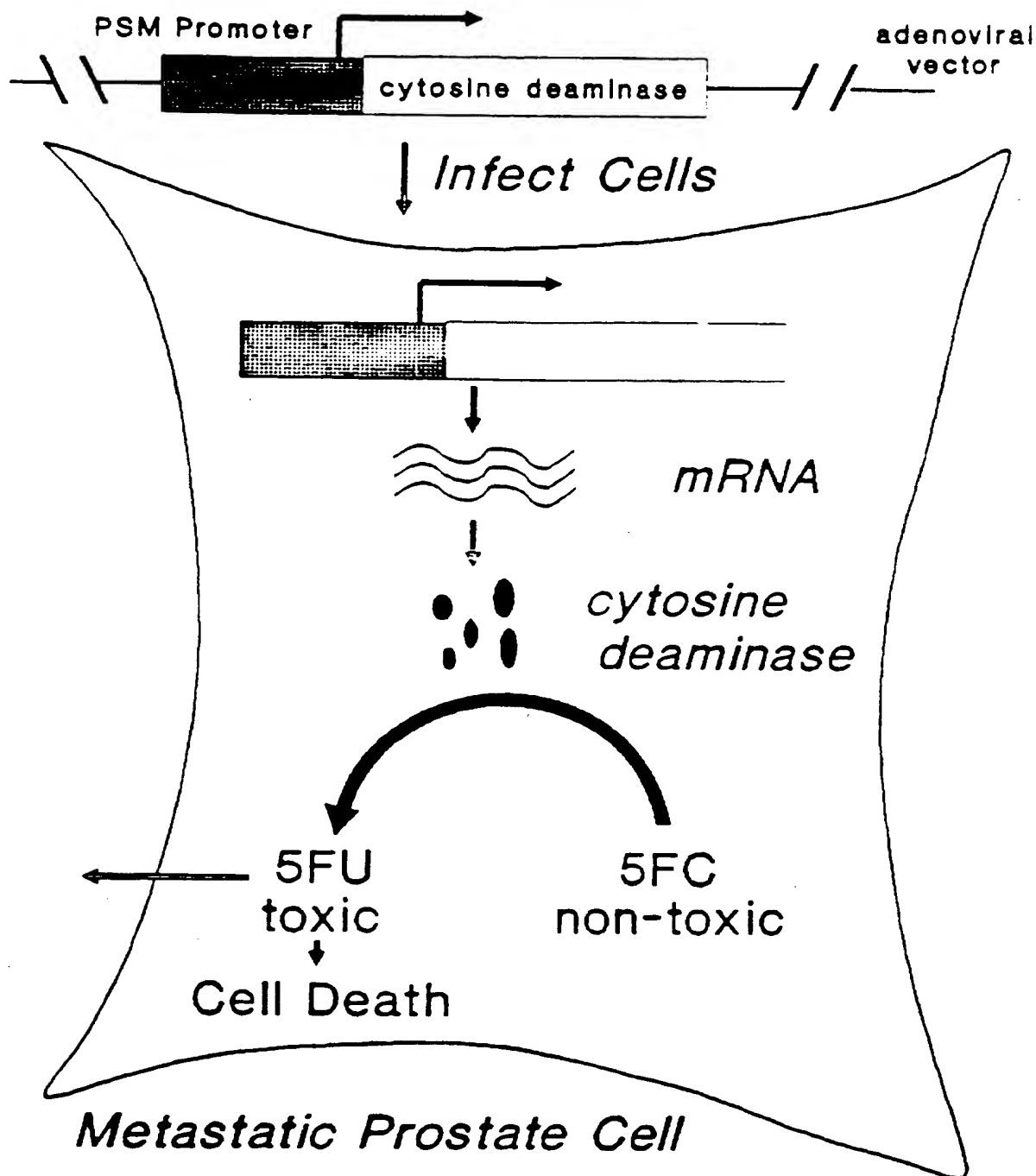## Prostate Specific Promoter: Cytosine Deaminase Chimera

FIGURE 58A

```
                10            20            30            40            50            6C
                 |             |             |             |             |             |
  1  GCGCCTTAAA AAAAAAAAAC TTTCTTGGAA AATGTCCAGC TCTTGCTTAA ATATAAAAT
     CGCGGAATTT TTTTTTTTG AAAGAACCTT TTACAGGTCG AGAACGAATT TATATTTTA

 61  GAAAGGAAGA AAGAGACTCT CCTCTCTCCA CTCCTATAAT TATGAGGAAC TTTTATTCAA
     CTTTCCTTCT TTCTCTGAGA GGAGAGAGGT GAGGATATTA ATACTCCTTG AAAATAAGTT

121  CTCTGAAATT CTATACAATC TCTACAATAC TCTACTGAAT AAAAGCAGAG CAGAAAAGC
     GAGACTTTAA GATATGTTAG AGATGTTATG AGATGACTTA TTTTCGTCTC GTCTTTTCG

181  TGCGCTTTTT TTCCATAGTC GGGAATGCTT GTCATCAGTG TAAATCACCA CCGCGCCTT
     ACGCGAAAAA AAGGTATCAG CCCTTACGAA CAGTAGTCAC ATTAGTGGT GGCGCGGGAA

241  TTTCCTAAAG AATATTATTG TTATTAATAA ACATGTAGGG TATTATCCTC CACTTACATT
     AAAGGATTTC TTATAATAAC AATAATTATT TGTACATCCC ATAATAGGAG GTGAATGTAA

3C1  ACAAAACCAT TTTTTAAAGC CGGGCGTGGT GGCTCACGCC TGTAATCCCA GCACTTTGGG
     TGTTTTGGTA AAAAATTTCG GCCCGCACCA CCGAGTGCGG ACATTAGGGT CGTGAAACCC

3£1  AGGCCCAGAC AGGCGGATCA CGAAGTCGAG AAATCGAGAC CATCCTGGCC AACATGGTGA
     TCCGGGTCTG TCCGCCTAGT GCTTCAGCTC TTTAGCTCTG GTAGGACCGG TTGTACCACT

421  AACCCCATCT CTACTAAAAA TACAAAAATT AGCTGGGCGT GGTGGCGGGC TCCTGTAGTC
     TTGGGGTAGA GATGATTTT ATGTTTTAA TCGACCCGCA CCACCGCCCG AGGACATCAG

481  CCAGCTACTC AGGAGGCTGA GGCAGGAGAA TGGCTTGAAC CGGGGAGGCG GAGGTTGCAG
     GGTCGATGAG TCCTCCGACT CCGTCCTCTT ACCGAACTTG GCCCCTCCGC CTCCAACGTC

541  TCAGCCAAGA TAGCGCCACT GCACTGGAGC TTGGTGACAG AGTGAGACTC CCTCAAGAAA
     AGTCGGTTCT ATCGCGGTGA CGTGACCTCG AACCACTGTC TCACTCTGAG GGAGTTCTTT

601  GAAAGGAAGG GAAGGGAAAG GGAAGGAAGG GGAGGGGAAG GGAGGGGAGG GGAGGGGAGG
     CTTTCCTTCC CTTCCCTTTC CCTTCCTTCC CCTCCCCTTC CCTCCCCTCC CCTCCCCTCC

661  AAAGAAAAGA ATACTGGAAC TTGTTGAAGG CAGAGACTTT ATTTTCATAT CCCGGCTATG
     TTTCTTTTCT TATGACCTTG AACAACTTCC GTCTCTGAAA TAAAAGTATA GGGCCGATAC

721  TCTGGCTACT GTCTTACGTA ATAGATATAA AATCAATCTT GGTTGGATTA ACCAGAAGAA
     AGACCGATGA CAGAATGCAT TATCTATATT TTAGTTAGAA CCAACCTAAT TGGTCTTCTT
```

FIGURE 58B

```
 781  TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGTAATCA  GCTTGGACAG
      ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGGT CCCCATTAGT CGAACCTGTC

 841  GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
      CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGGTCACG

 901  CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAGT
      GTGTTCGTTT GAGGTATTTC CATAGGACAC GACTTATCTC TGACATCTCA CCATGTTTCA

 961  AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
      TTCTGTCTGT AATATAATTC AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021  AAATTTCAGT TTTACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
      TTTAAAGTCA AAATGGTACA CATTTAGTCC TTCTCATTAT CTTGTTTGGA ACTTCCCAGG

1081  CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
      GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141  AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
      TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTTATCCTT GTAATCCTTT

1201  GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATTCCCTTC CTTCCTGCCC
      CTATATCATG TAAGTCCTAA AACAATCTTT CTCTACTTCT TTAAGGGAAG GAAGGACGGG

1261  TAGGTCATCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTTCA
      ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAAAGGG TTTAAAAAGT

1321  CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTC
      GAAACGAGTC TTTCAGATGT AGCTTCGTGG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381  CACTTAACTC ATACTGTGCT CTCCGTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
      GTGAATTGAG TATGACACGA GAGGGAAAGA GTTTCGTTTG ACAAACGATA AGGAACTTAT

1441  CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGCCCCTAAT GTTTCTTCTC
      GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGGTA CCGGGGATTA CAAAGAAGAG

1501  ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTTCT GTTAAAAGCA GTGCTTCCAT
      TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTTCGT CACGAAGGTA

1561  AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA
      TTTCATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTTGTGT CAAATAAAAT

1621  TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
      ATTTCGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATAATTCT TAAATATCGT

1681  GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
      CCCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTTCTAACT AAAATATAGA

1741  ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
      TAATGCATTC TGTCATCGGT CTGTATCGGC CCTATACTTT TATTTCAGAG ACGGAAGTTG

1801  AAGTTCCAGT ATTCTTTTCT TTCCTCCCCT CCCCTCCCCT CCCTTCCCCT CCCCTTCCTT
      TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAAGGGGA GGGGAAGGAA

1861  CCCTTTCCCT TCCCTTCCTT TCTTTCTTGA GGGAGTCTCA CTCTGTCACC AGGCTCCAGT
      GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCCTCAGAGT GAGACAGTGG TCCGAGGTCA
```

FIGURE 58C

```
1921  GCAGTGGCGC  TATCTTGGCT  GACTGCAACC  TCCGCCTCCC  CGGTTCAAGC  GATTCTCCTG
      CGTCACCGCG  ATAGAACCGA  CTGACGTTGG  AGGCGGAGGG  GCCAAGTTCG  CTAAGAGGAC

1981  CCTCAGCCTC  CTGAGTAGCT  GGGACTACAG  GAGCCCGCCA  CCACGCCCAG  CTAATTTTTG
      GGAGTCGGAG  GACTCATCGA  CCCTGATGTC  CTCGGGCGGT  GGTGCGGGTC  GATTAAAAAC

2041  TATTTTTAGT  AGAGATGGGG  TTTCACCATG  TTGGCCAGGA  TGGTCTCGAT  TTCTCGACTT
      ATAAAAATCA  TCTCTACCCC  AAAGTGGTAC  AACCGGTCCT  ACCAGAGCTA  AAGAGCTGAA

2101  CGTGATCCGC  CTGTCTGGGC  CTCCCAAAGT  GCTGGGATTA  CAGGCGTGAG  CCACCACGCC
      GCACTAGGCG  GACAGACCCG  GAGGGTTTCA  CGACCCTAAT  GTCCGCACTC  GGTGGTGCGG

2161  CGGCTTTAAA  AAATGGTTTT  GTAATGTAAG  TGGAGGATAA  TACCCTACAT  GTTTATTAAT
      GCCGAAATTT  TTTACCAAAA  CATTACATTC  ACCTCCTATT  ATGGGATGTA  CAAATAATTA

2221  AACAATAATA  TTCTTTAGGA  AAAAGGGCGC  GGTGGTGATT  TACACTGATG  ACAAGCATTC
      TTGTTATTAT  AAGAAATCCT  TTTTCCCGCG  CCACCACTAA  ATGTGACTAC  TGTTCGTAAG

2281  CCGACTATGG  AAAAAAAGCG  CAGCTTTTTC  TGCTCTGCTT  TTATTCAGTA  GAGTATTGTA
      GGCTGATACC  TTTTTTTCGC  GTCGAAAAAG  ACGAGACGAA  AATAAGTCAT  CTCATAACAT

2341  GAGATTGTAT  AGAATTTCAG  AGTTGAATAA  AAGTTCCTCA  TAATTATAGG  AGTGGAGAGA
      CTCTAACATA  TCTTAAAGTC  TCAACTTATT  TTCAAGGAGT  ATTAATATCC  TCACCTCTCT

2401  GGAGAGTCTC  TTTCTTCCTT  TCATTTTTAT  ATTTAAGCAA  GAGCTGGACA  TTTTCCAAGA
      CCTCTCAGAG  AAAGAAGGAA  AGTAAAAATA  TAAATTCGTT  CTCGACCTGT  AAAAGGTTCT

2461  AAGTTTTTTT  TTTTTAAGGC  GCCTCTCAAA  AGGGGCCGGA  TTTCCTTCTC  CTGGAGGCAG
      TTCAAAAAAA  AAAAATTCCG  CGGAGAGTTT  TCCCCGGCCT  AAAGGAAGAG  GACCTCCGTC

2521  ATGTTGCCTC  TCTCTCTCGC  TCGGATTGGT  TCAGTGCACT  CTAGAAACAC  TGCTGTGGTG
      TACAACGGAG  AGAGAGAGCG  AGCCTAACCA  AGTCACGTGA  GATCTTTGTG  ACGACACCAC

2581  GAGAAACTGG  ACCCCAGGTC  TGGAGCGAAT  TCCAGCCTGC  AGGGCTGATA  AGCGAGGCAT
      CTCTTTGACC  TGGGGTCCAG  ACCTCGCTTA  AGGTCGGACG  TCCCGACTAT  TCGCTCCGTA

2641  TAGTGAGATT  GAGAGAGACT  TTACCCCGCC  GTGGTGGTTG  GAGGGCGCGC  AGTAGAGCAG
      ATCACTCTAA  CTCTCTCTGA  AATGGGGCGG  CACCACCAAC  CTCCCGCGCG  TCATCTCGTC

2701  CAGCACAGGC  CCGGGTCCCG  GGAGGCCGGC  TCTGCTCGCG  CCGAGATGTG  GAATCTCCTT
      GTCGTGTCCG  GGCCCAGGGC  CCTCCGGCCG  AGACGAGCGC  GGCTCTACAC  CTTAGAGGAA

2761  CACGAAACCG  ACTCGGCTGT  GGGCCACCGCC  CGCCGCCCGC  GCTGGCTGTG  CGCTGGGGCG
      GTGCTTTGGC  TGAGCCGACA  CCCGGTGGCGC  GCGGCGGGCG  CGACCGACAC  GCGACCCCGC

2821  CTGGTGCTGG  CGGGTGGCTT  CTTTCTCCTC  GGCTTCCTCT  TCGGTAGGGG  GGCGCCTCGC
      GACCACGACC  GCCCACCGAA  GAAAGAGGAG  CCGAAGGAGA  AGCCATCCCC  CCGCGGAGCG

2881  GGAGCAAACC  TCGGAGTCTT  CCCCGTGGTC  CCGCGCGTGCT  GGGACTCGCG  GGTCAGCTGC
      CCTCGTTTGG  AGCCTCAGAA  GGGGCACCAC  GGCGCCACGA  CCCTGAGCGC  CCAGTCGACG

2941  CGAGTGGGAT  CCTGTTGCTG  GTCTTCCCCA  GGGGCGGCGA  TTAGGGTCGG  GGTAATGTGG
      GCTCACCCTA  GGACAACGAC  CAGAAGGGGT  CCCCGCCGCT  AATCCCAGCC  CCATTACACC


3001  GGTGAGCACC  CCTCGAG
      CCACTCGTGG  GGAGCTC
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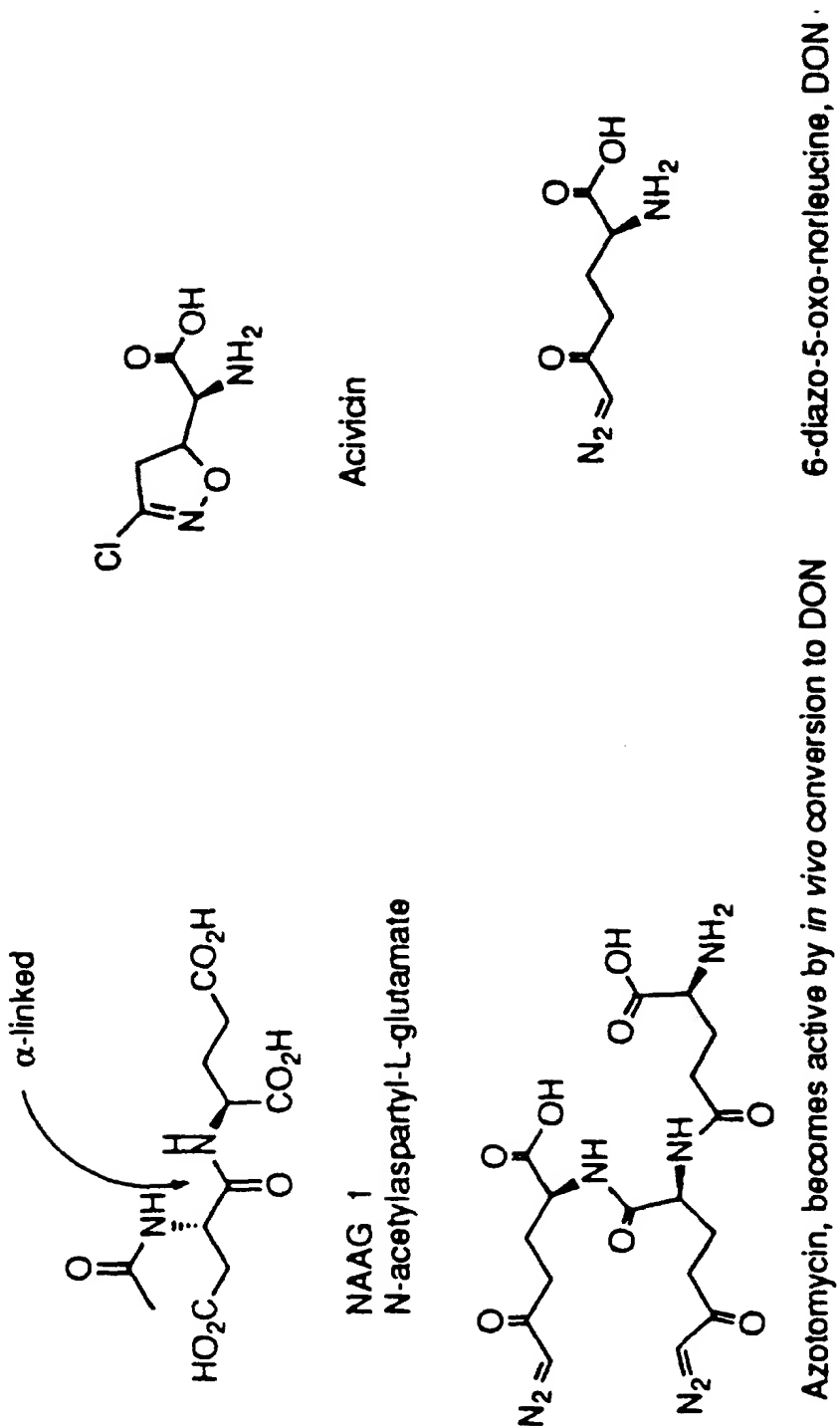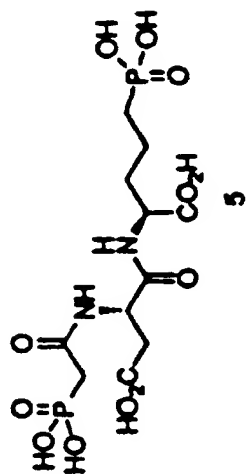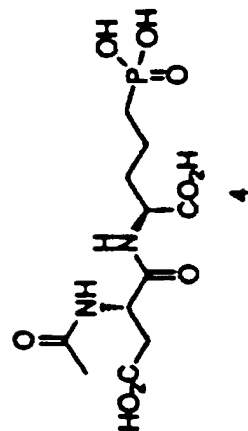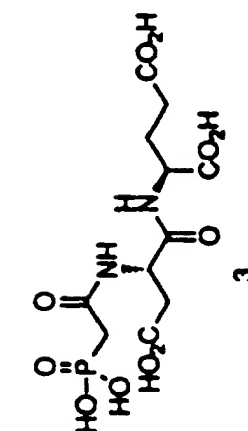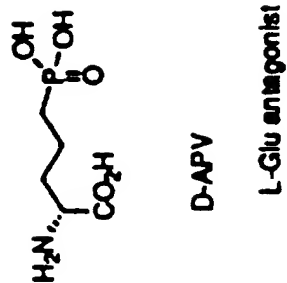
FIG. 59

Acivicin

6-diazo-5-oxo-norleucine, DON

NAAG 1
N-acetylaspartyl-L-glutamate

Azotomycin, becomes active by *in vivo* conversion to DON

FIG. 60

D-APV

L-Glu antagonist

5

PALA
N-phosphonoacetyl-L-aspartate
2

4

NAAG
N-acetylaspartyl-L-glutamate
1

3

FIG. 61

NAAG
Identical in all respects to an authentic
sample from Sigma.

Ac₂O = acetic anhydride
THF = tetrahydrofurane
DMF = N,N-dimethylformamide
PdC = palladium on charcoal
EtOAc = ethylacetate
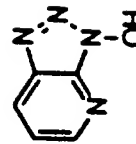
HOAt =
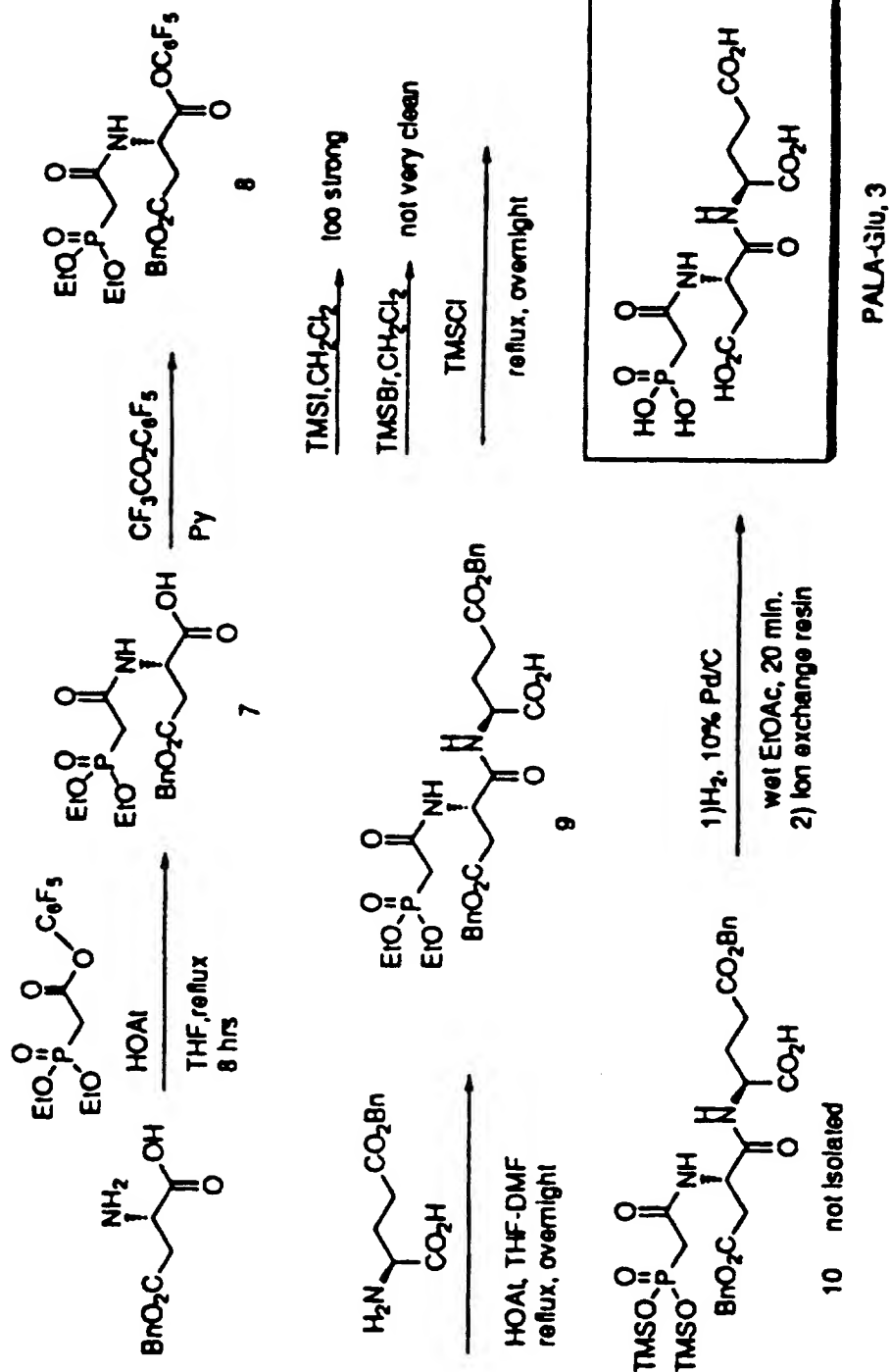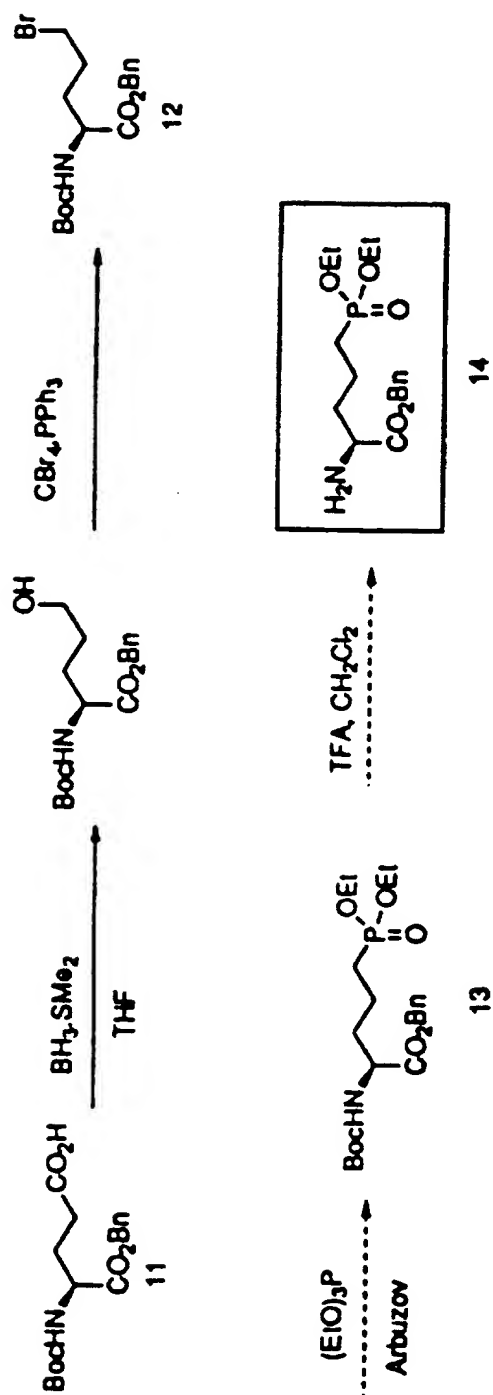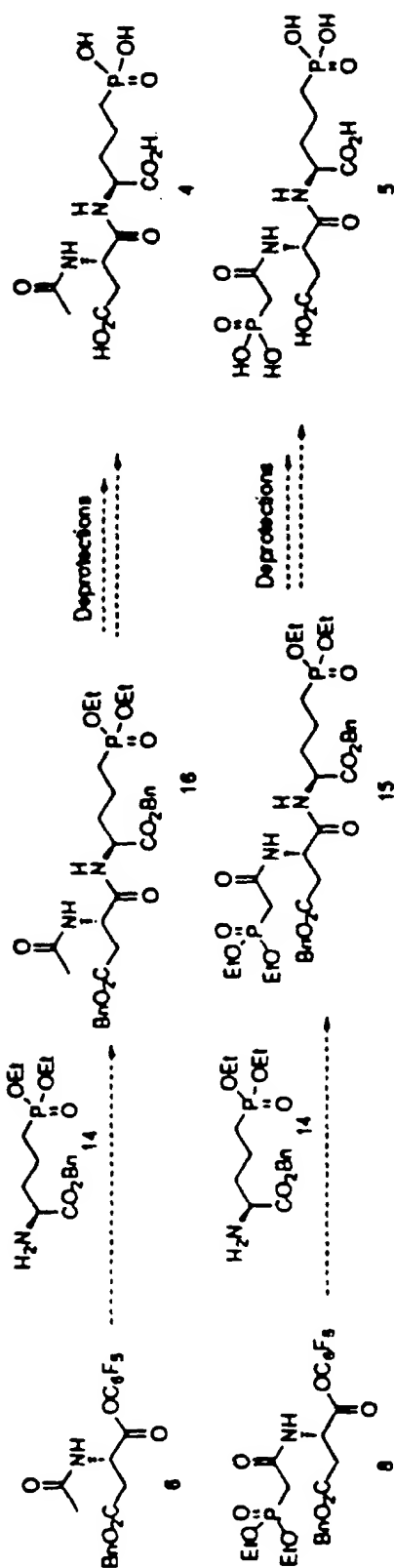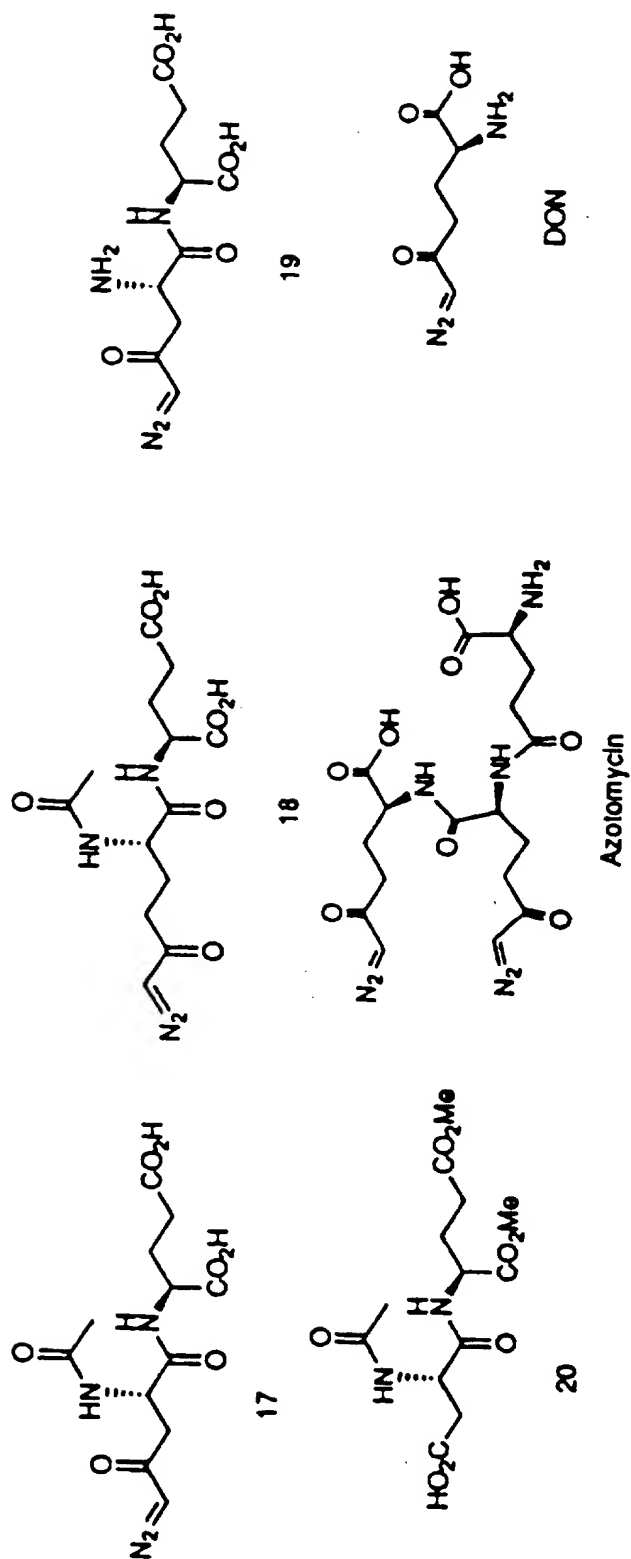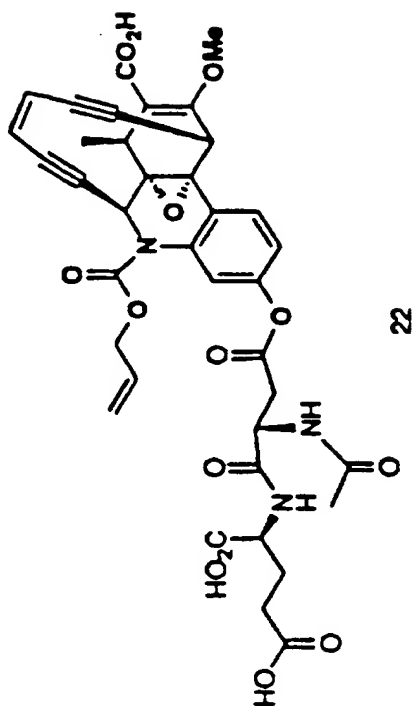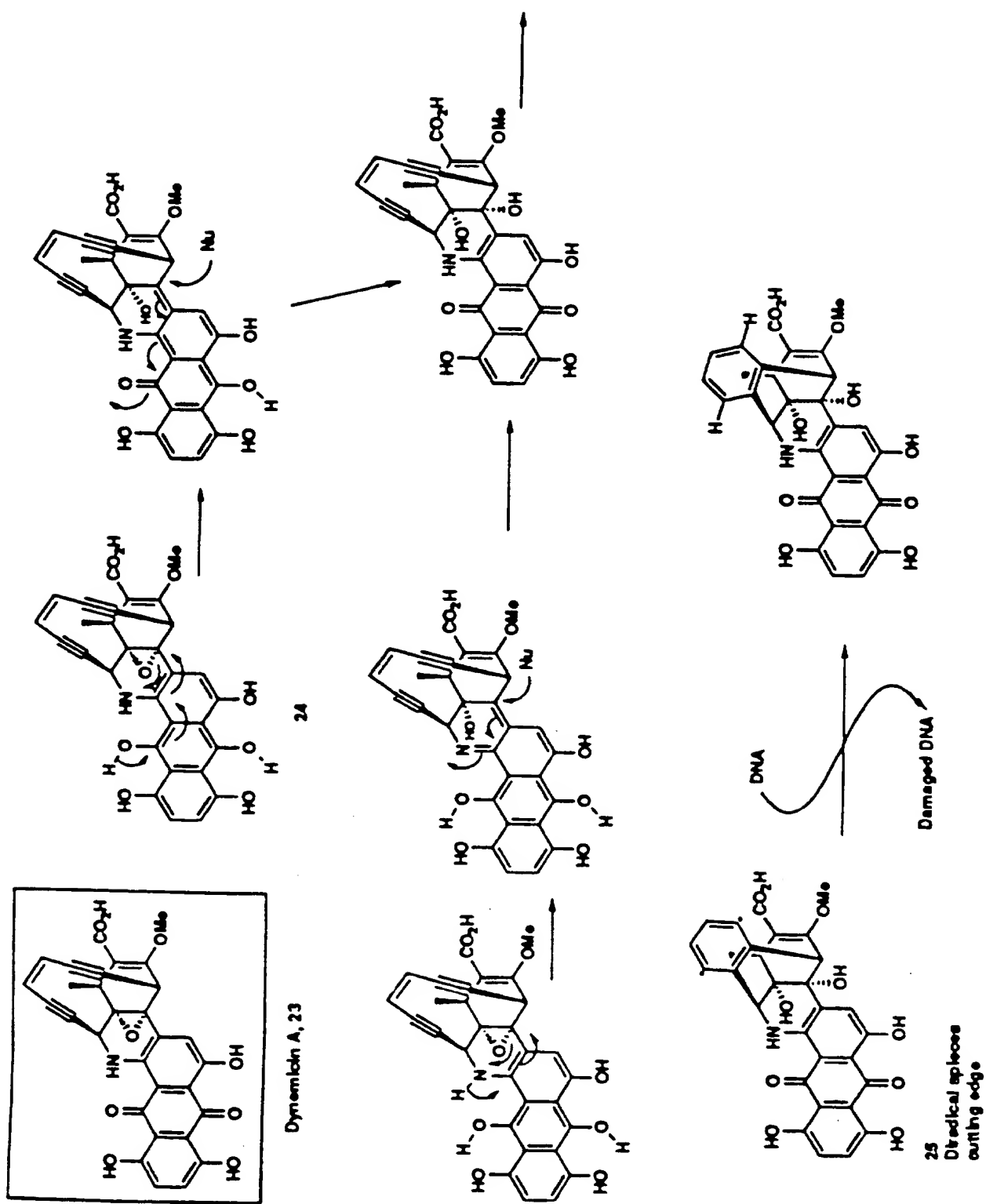
FIG. 62

FIG. 63

FIG. 64

FIG. 65

FIG. 66

FIG. 67

FIG. 68
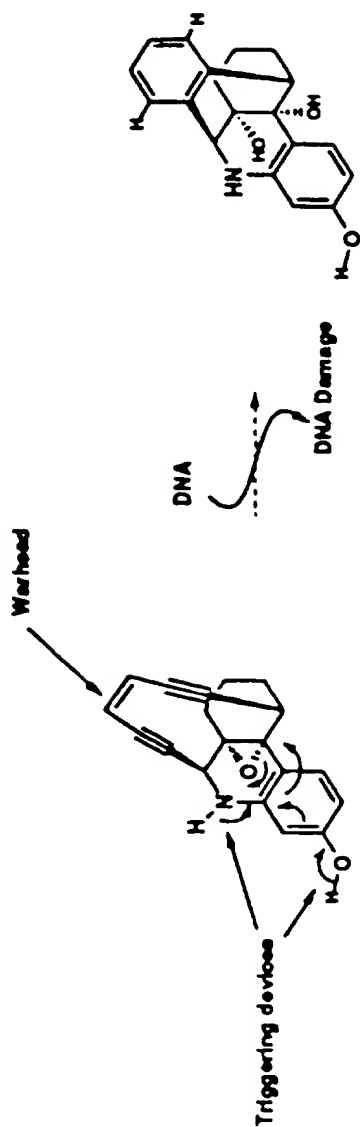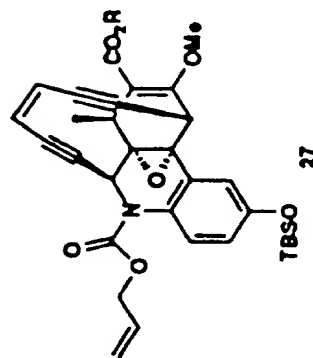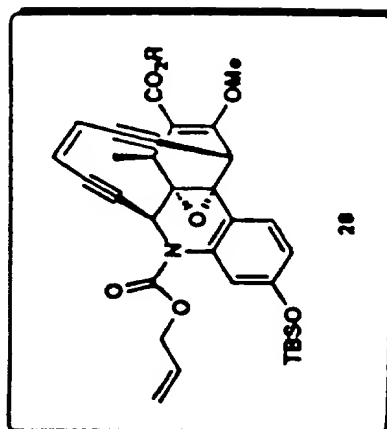
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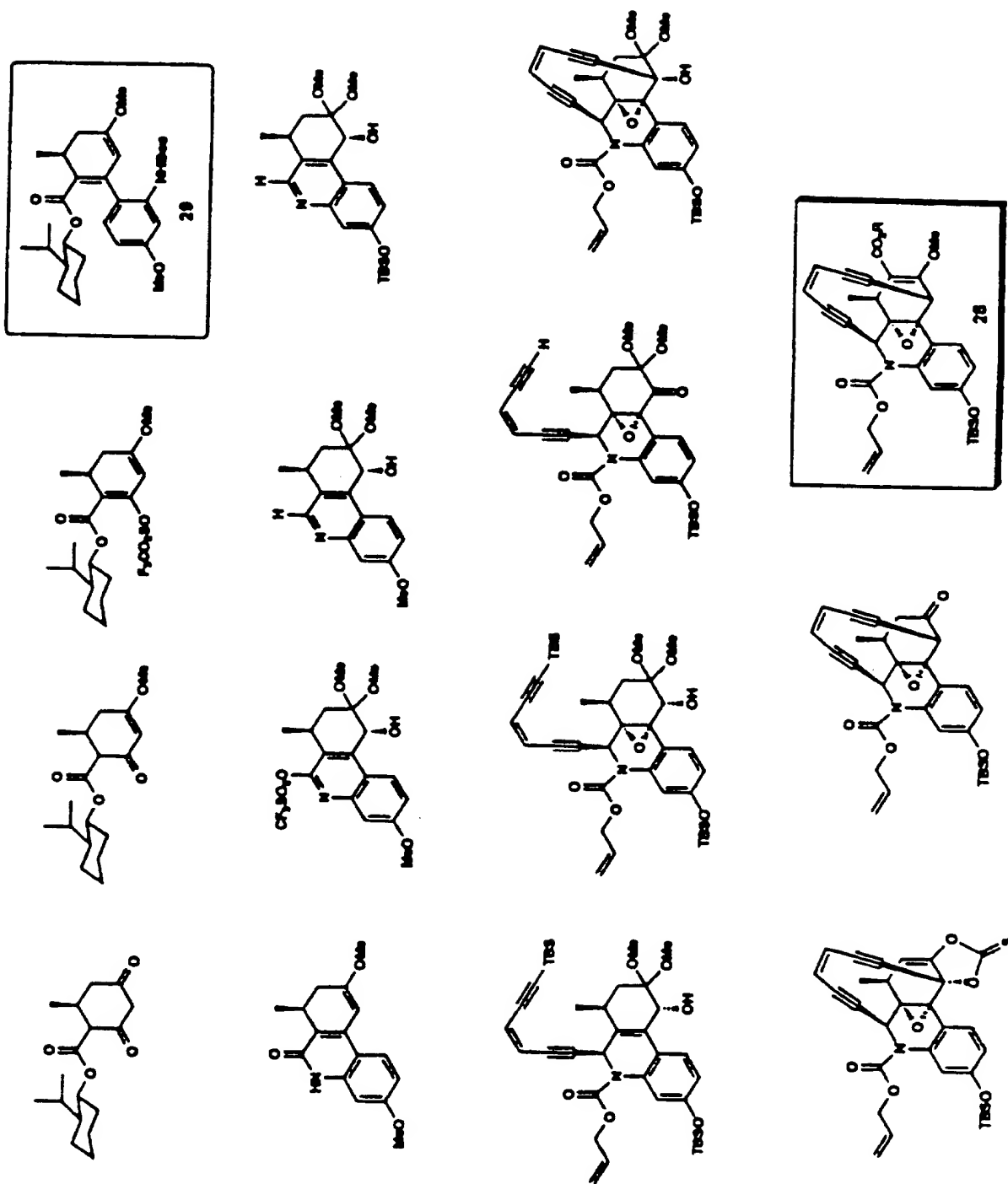
17 Steps for the optically active form 2

FIG. 70

FIG. 71

"THE GUIDANCE SYSTEM"

21

"THE SMART BOMB"

Peptidase

In this case

PSM

"THE TRIGGER"

30

26

"THE WARHEAD"

Prostat cancer cell damag

"THE WARHEAD"

Nitrogen trigger

Oxygen trigger
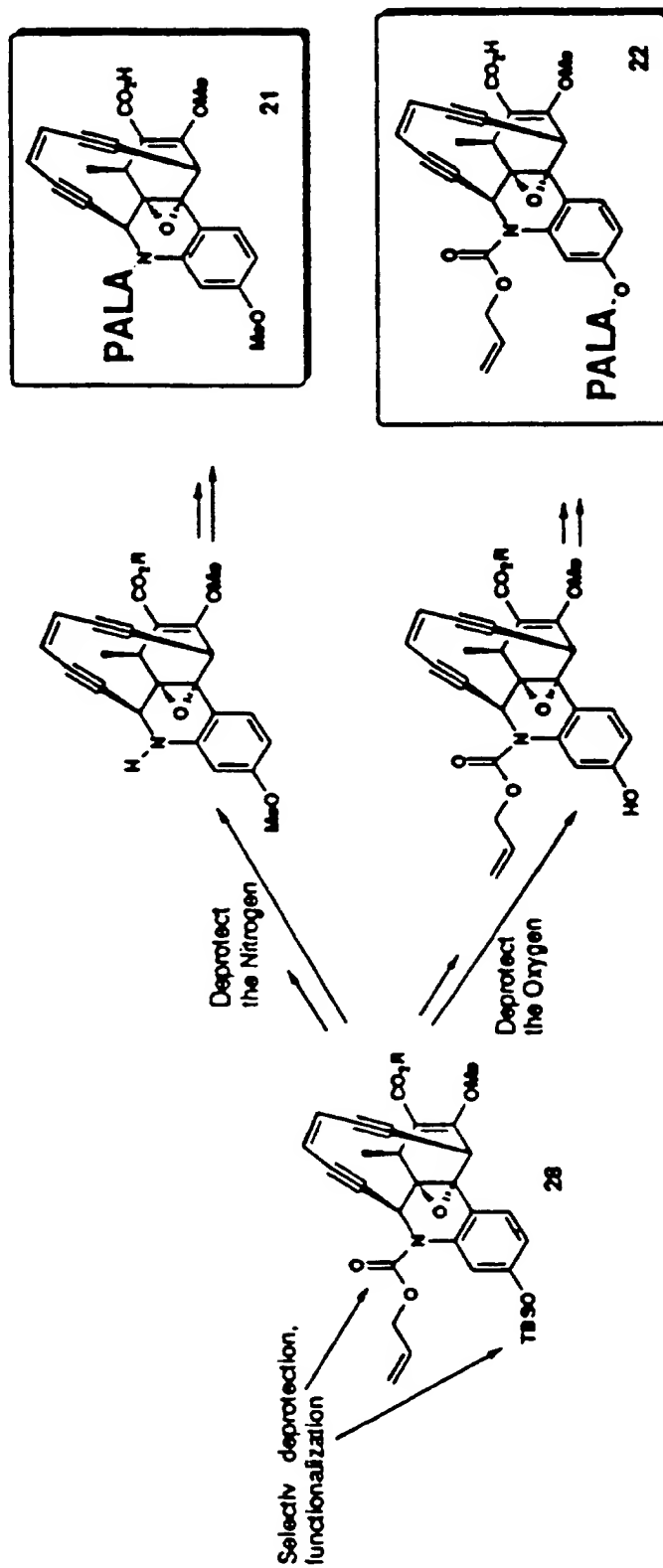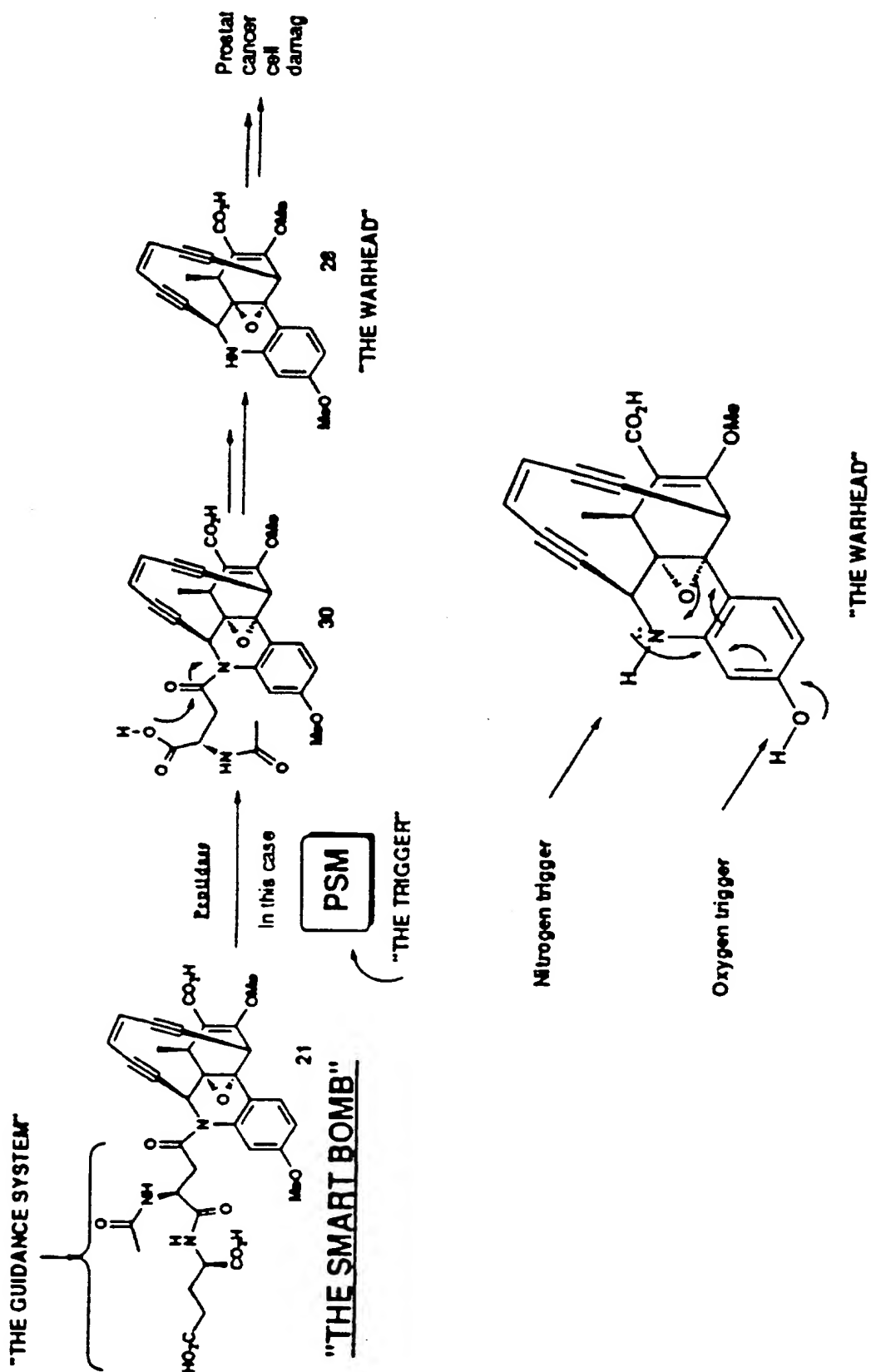
FIG. 72A

```
              10        20        30        40        50        60
              —         —         —         —         —         —
  1  TAGGGGGGCG CCTCGCGGAG AAACCTCGGA GTCTTCCCCG TGGTGCCGCG GTGCTGGGAC
     ATCCCCCCGC GGAGCGCCTC TTTGGAGCCT CAGAAGGGGC ACCACGGCGC CACGACCCTG

 61  TCGCGGGTCA GCTGCCGAGT GGGATCCTGT TGCTGGTCTT CCCCAGGGGC GGCGATTAGG
     AGCGCCCAGT CGACGGCTCA CCCTAGGACA ACGACCAGAA GGGGTCCCCG CCGCTAATCC

121  GTCGGGGTAA TGTGGGGTGA GCACCCCTCG AGTTAGGAGG AGGGTAGCTG GGAACGGCTGC
     CAGCCCCATT ACACCCCACT CGTGGGGAGC TCAATCCTCC TCCCATCGAC CCTTGCCACG

181  AGGGCTGAGT TCTCGACAAG CTGCTGGTAG GACAGTCACT CAGGTTGAGG GTAGAACTGA
     TCCCGACTCA AGAGCTGTTC GACGACCATC CTGTCAGTGA GTCCAACTCC CATCTTGACT

241  GAGAACCTGA AACTGGGCGT AGGAAGGTTC CAAGTGCTGG AGCCCTGCAA GACAGAGGAA
     CTCTTGGACT TTGACCCGCA TCCTTCCAAG GTTCACGACC TCGGGACGTT CTGTCTCCTT

301  GTTTTTTTT TGCTTTTGTT TTGTTTTGTT TTGTTTTGTT TTGTTTTGTT TGTTTGTTTG
     CAAAAAAAA ACGAAAACAA AACAAAACAA AACAAAACAA AACAAAACAA ACAAAACAAAC

361  TTTTTTTACC TCTCTGTGCA TTCTTTCTTC CTTGGAAGTA ACAGAGGCAA GCTTGGGAAC
     AAAAAAATGG AGAGACACGT AAGAAAGAAG GAACCTTCAT TGTCTCCGTT CGAACCCCTTG

421  TGTGTGAACC AGGTCAGCAA TCTGGACAGG TCTTTACCAG CGGGTCTTTT GCTGTTTTTC
     ACACACTTGG TCCAGTCGTT AGACCTGTCC AGAAATGGTC GCCCAGAAAA CGACAAAAAG

481  CTGGGTACTG ATTTGCAGAC TTGATCCAAC TTTCTAAGAA AAGCAGAACC ACACAGGCAA
     GACCCATGAC TAAACGTCTG AACTAGGTTG AAAGATTCTT TTCGTCTTGG TGTGTCCGTT

541  GCTCAGACTC TTTTATTAAA TTCCAGTTTT GACTTTGCCA CTTCTTAGTG GCCTTGAACA
     CGAGTCTGAG AAAATAATTT AAGGTCAAAA CTGAAACGGT GAAGAATCAC CGGAACTTGT
```
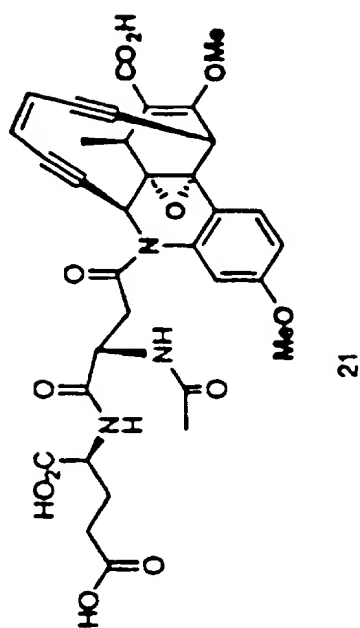
FIG. 72B

```
601  AGTTACCGAC  TCCCTCTCAG  CGTTAGTTAC  CCTATTTTAT  GATGAGGATA  ATATTATCTG
     TCAATGGCTC  AGGGAGAGTC  GCAATCAATG  GGATAAAATA  CTACTCCTAT  TATAATAGAC

661  CAAATTATTG  GTAATAGTAA  ATAATATAGC  ATGTAAATCT  CCTAGCACAG  TACTGGGATT
     GTTTAATAAC  CATTATCATT  TATTATATCG  TACATTAGA   GGATCGTGTC  ATGACCCTAA

721  TTCGCCACTT  TATTTCTTCT  TTTACCAAGA  TACTCCTCAT  TGGACTTTAA  TACACAGGAC
     AAGCGGTGAA  ATAAAGAAGA  AAATGGTTCT  ATGAGGAGTA  ACCTGAAATT  ATGTGTCCTG

781  TAGTCTAAGG  TATCACCAGG  TAGTCCACTC  CTGCTCGGAA  TTCTTGACCC  TCTTTCGGGA
     ATCAGATTCC  ATAGTGGTCC  ATCAGGTGAG  GACGAGCCTT  AAGAACTGGG  AGAAAGCCCT

841  TTTAGAAGAA  TAGGGCATGG  ACCAGATGGG  TTTAAACAAA  TTCAATATCT  TCCACTAGCT
     AAATCTTCTT  ATCCCGTACC  TGGTCTACCC  AAATTTGTTT  AAGTTATAGA  AGGTGATCGA

901  TCACCTTGGG  GTTGTTAAAA  GATTTTTGAA  CCACACACTG  TGCTCATAAC  AATCTTCATC
     AGTGGAACCC  CAACAATTTT  CTAAAAACTT  GGTGTGTGAC  ACGAGTATTG  TTAGAAGTAG

961  TCTTAAAAGG  ATTTTATTCT  TCCTGGTATT  GCCCTCACTC  TCATCCCTGT  ATTCCGTGCT
     AGAATTTTCC  TAAAATAGA   AGGACCATAA  CGGGAGTGAG  AGTAGGGACA  TAAGGCACGA
```

FIG. 72C

```
1021  CAGTGGCTCA CACAGAAGAG TCTTTATTG  ATGTCCGCCC CCCACCCACT AGGATTCTCT
      GTCACCGACT GTGTCTTCTC AAGAAATAAC TACAGGCGGG GGGTGGGTGA TCCTAAGAGA

1081  GCTCTCCCCT CCCCTACAG  GCCTCCATCC TCTTCATCCT GTTCATTTT  CAGATCTCAG
      CGAGAGGGGA GGGGGATGTC CGGAGGTAGG AGAAGTAGGA CAAGTAAAAA GTCTAGAGTC

1141  TTCAAGCATC TCGTCCTCAG TGTGGTGTTT CCTGATCCCT CACTCTAATC CAAGTCTTTC
      AAGTTCGTAG AGCAGGAGTC ACACCACAAA GGACTAGGGA GTGAGATTAG GTTCAGAAAG

1201  TGTTTATGC  ACAGGTGGAA TCTTATTTCC GTTTGCGTCC AATCATGTAT TTTAATATGC
      ACAAAATACG TGTCCACCTT AGAATAAAGG CAAACGCAGG TTAGTACATA AAATTATACG

1261  ATGTATATAT GTATCTGCAT TTGTATGCAT GCGATTAAGA ACTAGAAATA TTAATAATTG
      TACATATATA CATACACGTA AACATACGTA CGCTAATTCT TGATCTTATT AATTATTAAC

1321  GAAAGCTCCA TGAAAGCTGG TTGGGGACTA ATTTTGTAAC TACTTTATTC CCAGATCCTG
      CTTTCGAGGT ACTTTCGACC AACCCCTGAT TAAACATTG  ATGAAATAAG GGTCTAGGAC

1381  TAATTTCTCT AAATAAACCC TGGAATCTTG CCTTATCTCC TTCAGGTTAA AAGCCAACTG
      ATTAAGAGA  TTTATTTGGG ACCTTAGAAC GGAATAGAGG AAGTCCAATT TTCGGTTGAC

1441  CAAGGTCTAA TGACTGCAGG ATCTAGCTAT CCATTGTTTC TGGCCGCCTA TGCGTGCACT
      GTTCCAGATT ACTGACGTCC TAGATCGATA GGTAACAAAG ACCGGCGGAT ACGCACGTGA

1501  GGGTGTCTGG CAGAGAGGCT GGGTAAAATTG TAGTTTCATT GTAGCTGTCT GACTTGGATT
      CCCACAGACC GTCTCTCCGA CCCATTTAAC ATCAAAGTAA CATCGACAGA CTGAACCTAA

1561  TCTCACGCCT ACTTCACTGG AAACGCAAAC TCTCACAGCA TTTGTTTTA  GTTTCAGAAT
      AGAGTGCGGA TGAAGTGACC TTTGCGTTTG AGAGTGTCGT AAAACAAAAT CAAAGTCTTA

1621  CAGAGCAAAT TAGAAGTCTG AATTCCTTC  AACACTTGGA AATAATTTAT TTATTTGAAA
      GTCTCGTTTA ATCTTCAGAC TTAAGGAAG  TTGTGAACCT TTATTAAATA AATAAACTTT

1681  TATATTCATA ATTAATTCGT TATTAAAATG TATTAAATGC TTATTTGAGT CAGCAGAGGA
      ATATAAGTAT TAATTAAGCA ATATTTTTAC ATAATTTACG AATAAACTCA GTCGTCTCCT
```

FIG. 72D

```
1741  AGATAGAAAC  TTTATGAAAG  TAGAAGGTGG  ATCTCCTTTT  TGCCTTCATT  TTCAGAAACAT
      TCTATCTTTG  AAATACTTTC  ATCTTCCACC  TAGAGGAAAA  ACGGAAGTAA  AAGTCTTGTA

1801  CTCGTTTACA  CCCATTAGTT  GAAACATTAA  TGTCATTTTA  TTTTCGTCCT  GATTATCTCA
      GAGCAAATGT  GGGTAATCAA  CTTTGTAATT  ACAGTAAAAT  AAAAGCAGGA  CTAATAGAGT

1861  TAAAACATTT  CTTAGAATAA  CAGCAATACC  TATCATTGAA  GTTGGATAAG  AAATATTTTG
      ATTTTGTAAA  GAATCTTATT  GTCGTTATGG  ATAGTAACTT  CAACCTATTC  TTTATAAAAC

1921  CAATTGGTTT  GCAACTTAAA  AATCTGTTTG  CATGACTCTT  TTTCAGTGAA  AGTAGGCAAG
      GTTAACCAAA  CGTTGAATTT  TTAGACAAAC  GTACTGAGAA  AAAGTCACTT  TCATCCGTTC

1981  AGAAATTAAA  ATTCAGAAAT  ATCTCACCTA  ATGTCAGAGG  TAATATTGAT  AATTTGTGTT
      TCTTTAATTT  TAAGTCTTA   TAGAGTGGAT  TACAGTCTCC  ATTATAACTA  TTAAACACAA

2041  TTACAAATAA  TACATACAAC  AATAATGAAA  AATAAGTCCT  ATCTATAGGC  TCGTATCTCA
      AATGTTTATT  ATGTATGTTG  TTATTACTTT  TTATTCAGGA  TAGATATCCG  AGCATAGAGT

2101  TGCCTATTTT  TGGATGTATT  TTTCA
      ACGGATAAAA  ACCTACATAA  AAAGT
```

FIG. 73A

```
              10         20         30         40         50         60
              |          |          |          |          |          |
  1  TGAAAAATAC ATCAAAAATA GGCATGAGAT ACGAGCCTAT AGATAGGACT TATTTTTTAT
     ACTTTTTATG TAGTTTTTAT CCGTACTCTA TGCTCGGATA TCTATCCTGA ATAAAAAATA

 61  TATTGTTGTA TGTATTATTT GTAAAACACA AATTATCAAT ATTACCTCTG ACATTAGGTG
     ATACAACAAT ACATAATAAA CATTTTGTGT TTAATAGTTA TAATGGAGAC TGTAATCCAC

121  AGATATTCTG AATTTTAATT TCTCTTGCCT ACTTTCACTG AAAAAGAGTC ATGCAAACAG
     TCTATAAGAC TTAAAATTAA ACAGAACGGA TGAAAGTGAC TTTTTCTCAG TACGTTTGTC

181  ATTTTTAAGT TGCAAACCAA TTGCAAAATA TTTTTTTATC CAACTTCAAT GATAGGTATT
     TAAAAATTCA ACGTTTGGTT AACGTTTTAT AAAAAAATAG GTTGAAGTTA CTATCCATAA

241  GCTGTTAATT CTAAGATATG CATTAATTGT TTCAACTAAT GGGTGTCAAA CGAGATGTTC
     CGACAATTAA GATTCTATAC GTAATTAACA AAGTTGATTA CCCACAGTTT GCTCTACAAG

301  TGAAAATGAA GGCAAAAAGG AGATCCACCT TCTACTTCA TAAAGTTTCT ATCTTCCTCT
     ACTTTTACTT CCGTTTTTCC TCTAGGTGGA AGATGAAAGT ATTTCAAAGA TAGAAGGAGA

361  GCTGACTCAA ATAAGCATTT AATACATTTT ATAACGAATT AATTATGAAT ATATTTCAAA
     CGACTGAGTT TATTCGTAAA TTATGTAAAA TATTGCTTAA TTAATACTTA TATAAGTTTT

421  TAAATAAATT ATTTCCAAGT GTTGAAGGAA ATTCAGACTT CTAATTGCT CTGATTCTGA
     ATTTATTTAA TAAAGGTTCA CAACTTCCTT TAAGTCTGAA GATTAAACGA GACTAAGACT
```

FIG. 73B

```
481  AACTAAAACA  AATGCTCTGT  GAGAGTTTGC  GTTTCCAGTG  AAGTAGCGTG  AGAAATCCAA
     TTGATTTTGT  TTACGAGACA  CTCTCAAACG  CAAAGGTCAC  TTCATCGCAC  TCTTTAGGTT

541  GTCAGACAGC  TACATGAAAC  TACATTTACC  AGCTCTCTGC  CAGACACCAG  TGCACGATAG
     CAGTCTGTCG  ATGTACTTTG  ATGTAAATGG  TCGAGAGACG  GTCTGTGGTC  ACGTGCTATC

601  CGCAGAACAT  GTAGCTAGAT  CTCAGTCATA  GCTNNNNNNN  NNNNNNNNN  AGACCTTGCA
     GCGTCTTGTA  CATCGATCTA  GAGTCAGTAT  CGANNNNNNN  NNNNNNNNN  TCTGGAACGT

661  GTTGGCTTTT  AACCTGAAGG  AGATAAGGCA  AGATTCCAGG  GTTTATTTAG  AGAAATTACA
     CAACCGAAAA  TTGGACTTCC  TCTATTCCGT  TCTAAGGTCC  CAAATAAATC  TCTTTAATGT

721  GGATCTGGGA  ATAAAGTAGT  TACAAAATTA  GTCCCCAACC  AGCTTTCATG  GAGCTTTCAA
     CCTAGACCCT  TATTTCATCA  ATGTTTTAAT  CAGGGGTTGG  TCGAAAGTAC  CTCGAAAGTT
```

FIG. 73C

```
 781  TTATTAATTA  TTCTAGTTCT  TAATCGGATG  CATACAATGC  ACATACATAT  ATACATGCAT
      AATAATTAAT  AAGATCAAGA  ATTAGCGTAC  GTATGTTACG  TGTATGTATA  TATGTACGTA

 841  ATTAAATAC   ATGATTGGAC  GCAAACGGAA  ATAAGATTCC  ACCTGTGCAT  AAAACAGAAA
      TAATTTTATG  TACTAACCTG  CGTTGCCTT   TATTCTAAGG  TGGACACGTA  TTTTGTCTTT

 901  GACTTGGTTA  GAGTGAGGGA  TCAGGAAACA  CCACACTGAG  GACGAGATGN  NNNNNNNNNN
      CTGAACCAAT  CTCACTCCCT  AGTCCTTGT   GGTGTGACTC  CTGCTCTACN  NNNNNNNNNN

 961  NTAGTGGGTG  GGGGGCCGAC  ATCAATAAAG  AACTCTTCTG  TGTCAGCCAC  TGAGCACGGA
      NATCACCCAC  CCCCGCCTG   TAGTTATTTC  TTGAGAAGAC  ACAGTCGGTG  ACTCGTGCCT

1021  ATAAAGGGAT  GAGAGTGAGG  GCAANTACCA  GAAGAATAAA  ATCCTTTTAA  GAGATGAAGA
      TATTTCCCTA  CTCTCACTCC  CGTTNATGGT  CTTCTTATTT  TAGGAAAATT  CTCTACTTCT

1081  TTGTTATGAG  CACAGTGTGT  GGNTTCAAAA  ATCTTTTAAC  AACCCCAAGG  TCAAGCTAGT
      AACAATACTC  GTGTCACACA  CCNAAGTTTT  TAGAAAATTG  TTGGGGTTCC  ACTTCGATCA

1141  TGGAAGATAT  TTGAATTTGT  TTAAACCCAT  CTGGTCCTAG  CCCTATTCTT  TGAATCCCGA
      ACCTTCTATA  AACTTAAACA  AATTTGGGTA  GACCAGGATC  GGGATAAGAA  ACTTAGGGCT
```

FIG. 73D

```
1201 AAGAGGGTCA AGAATTCCGA GCAGGAGTGG ACTACCTGGT GATACCTTAG ACTAGTCCTG
     TTCTCCCAGT TCTTAAGGCT CGTCCTCACC TGATGGACCA CTATGGAATC TGATCAGGAC

1261 TGTATTAAAG TCCAATGAGG AGTATCTTGG TAAAATAATA AATAAAGTCC CGAAAATCCC
     ACATAATTTC AGGTTACTCC TCATAGAACC ATTTTATTAT TTATTTCAGG GCTTTTAGGG

1321 AGTACTGTGC TAGGAGATTT ACATGCTATA TTATTTACTA TNNNNNNNNT AATTTGCAGA
     TCATGACACG ATCCTCTAAA TGTACGATAT AATAAATGAT ANNNNNNNNA TTAAACGTCT

1381 TAATATTATC CTCATCATAA AATAGGGTAA CTAACGCTGA GAGGGACTCG GTAACTTGTT
     ATTATAATAG GAGTAGTATT TTATCCCATT GATTGCGACT CTCCCTGAGC CATTGAACAA

1441 CAAGGCCACT AAGAAGTGGC AAAGTCAAAA CTGGAATTTT AATAAAAGAG TCTAGCTTGC
     GTTCCGGTGA TTCTTCACCG TTTCAGTTTT GACCTTAAAA TTATTTTCTC AGATCGAACG

1501 CTGTGTGGTT CTGCTTTTCT TAGAAAGTTG GANNAAGTCT CANATCAGTA CCCAGGAAAA
     GACACACCAA GACGAAAAGA ATCTTTCAAC CTNNTTCAGA GTNTAGTCAT GGGTCCTTTT

1551 ACAGCAAAAG ACCCGCTGGT AAAGACCTGT CCAGATTGCT GACCTGGTTC ACACANTTCC
```

FIG. 73E

```
    TGTCGTTTTC TGGGCGACCA TTTCTGGACA GGTCTAACGA CTGGACCAAG TGTGTNAGG

1621 AAGCTTGCCT CTGTTACTTC CAAGGAAGAA AGAATGCACA GAGAGGTAAA AAAACAAACA
     TTCGAACGGA GACAATGAAG GTTCCTTCTT TCTTACGTGT CTCTCCATTT TTTTGTTTGT

1681 AACCAAAACAA AACAAAACAA AACAAAACAA AAGCAAAAAA AAACTTCCTC
     TTGGTTTGTT TTGTTTTGTT TTGTTTTGTT TTCGTTTTTT TTTGAAGGAG

1741 TGTCTTGCAG GGCTCCAGCA CTTGGAACCT TCCTACGTCC TANTTTCAGG TTCTCTCAGT
     ACAGAACGTC CCGAGGTCGT GAACCTTGGA AGGATGCAGG ATNAAAGTCC AAGAGAGTCA

1801 TCTACCCTCA ACCTGAGTGA CTGTCCTACC AGCAGCTTGT CGAGAACTCA GCCCTGCACC
     AGATGGGAGT TGGACTCACT GACAGGATGG TCGTCGAACA GCTCTTGAGT CGGGACGTGG

1861 GTTCCCAGCT ACCCTCCTCC TAACTCGAGG GGTGCT
     CAAGGGTCGA TGGGAGGAGG ATTGAGCTCC CCACGA
```

FIG. 74A

```
         10        20        30        40        50        60
         |         |         |         |         |         |
  1  GGATTCTGTT GAGCCCTAGC TCATTATGAT GTCCTGTTGT CCTACCCCAA TAAGACTCAT
     CCTAAGACAA CTCGGGATCG AGTAATACTA CAGGACAACA GGATGGGGTT ATTCTGAGTA

 61  CCCAACTACA TCTCAATAAT TAATGAAGAT GGAAATGAGG TAAAAATAA ATAAAATAAT
     GGGTTGATGT AGAGTTATTA ATTACTTCTA CCTTTACTCC ATTTTTATT TATTTATTTA

121  AAAGAAACA TTCCCCCCCA TTTATTATTT TTTCAAATAC CTTCTATGAA ATAATGTTCT
     TTTCTTTGT AAGGGGGGT AAATAATAAA AAAGTTTATG GAAGATACTT TATTACAAGA

181  ATCCCTCTCT AAATATTAAT AGAAATCAAT ATTATTGGAA CTGTGAATAC CTTTAATATC
     TAGGGAGAGA TTTATAATTA TCTTTAGTTA TAATAACCTT GACACTTATG GAAATTATAG

241  TCATTATCCG GTGTCAACTA CTTTCCTATG ATGTTGAGTT ACTGGGTTTA GAAGTCGGGA
     AGTAATAGGC CACAGTTGAT GAAAGCATAC TACAACTCAA TGACCCAAAT CTTCAGCCCT

301  AATAATGCTG TAAANNNNNN AGTTAGTCTA CACACCAATA TCAAAATATGA TATACTTGTA
     TTATTACGAC ATTTNNNNNN TCAATCAGAT GTGTGGTTAT AGTTTATACT ATATGAACAT

361  AACCTCCAAG CATAAAAGA GATACTTTAT AAAGAGGTT CTTTTTTCT TTTTTTTTT
     TTGGAGGTTC GTATTTTCT CTATGAAATA TTTCTCCAA GAAAAAAGA AAAAAAAAA
```

FIG. 74B

```
421  TCCAGATGGA  GTTTCACTCC  TGTCAGGCAG  GCNGAGTGCA  GTGGTGCCAT  CTCGGCTCAC
     AGGTCTACCT  CAAAGTGAGG  ACAGTCCGTC  CGNCTCACGT  CACCACGGTA  GAGCCGAGTG

481  TGCAACCTCC  ACCTCCCATG  TTCAAGGGAT  TCTCCTTCCT  CAGTCTCCTG  AGTAGCTGGG
     ACGTTGGAGG  TGGAGGGTAC  AAGTTCCCTA  AGAGGAAGGA  GTCAGAGGAC  TCATCGACCC

541  ATTACAGGTG  TGCACCACCA  CACCCAGCTA  ATTTTTGTAT  TTTTAATAGA  GACAGGGTTT
     TAATGTCCAC  ACGTGGTGGT  GTGGGTCGAT  TAAAAACATA  AAAATTATCT  CTGTCCCAAA

601  CATCGATGTT  GGCCAGGCTA  GTCTCGAACT  CCTGACCTCT  AGGTGATCCA  CCCGCCTCAG
     GTAGCTACAA  CCGGTCCGAT  CAGAGCTTGA  GGACTGGAGA  TCCACTAGGT  GGGCGGAGTC

661  CCTCCCAAAG  TTGTAGAATT  ACACGTGTGA  GGCACTGCTC  TGGCCAGGAG  ATACATTTT
     GGAGGGTTTC  AACATCTTAA  TGTGCACACT  CCGTGACGAG  ACCGGTCCTC  TATGTAAAA

721  GATAGGTTTA  ATTTATAAAG  ACACTGCACA  GATTGGCACT  TGCTGGGAAA  TCACGGATCCA
     CTATCCAAAT  TAAATATTTC  TGTGACGTGT  CTAACCTCA  ACGACCCTTT  AGTGCTAGGT
```

FIG. 74C

```
781  GTATGCATTT GACCCAGCAA TTTTATTGG TACTTAATGA TTATATCTCA ATTGATCAGG
     CATACGTAAA CTGGGTCGTT AAAATAACG ATGAATTACT AATATATAGT TAACTAGTCC

841  TTGAACTCTG TGCGAAGAAT TTGTGTGTGG ACATTGGAGA GGACAGTTTG GAGGCAAGGT
     AACTTGAGAC ACGGTTCTTA AACACACACC TGTAAACTCT CCTGTCAAAC CTCCGTTCCA

901  ATTTTAGTAG ATTTAAAGAA TTTGAATCTT GTTTGCAAGT TGGGGCATAT ACTGAGAAAG
     TAAAATCATC TAAATTTCTT AAACTTAGAA CAAACGTTCA ACCCCGTATA TGACTCTTTC

961  AGAAGACAAT GCAGATAAAT TGATATATTT ATTATGATGT ATGTTCAATA TGAAAGATCA
     TCTTCTGTTA CGTCTATTTA ACTATATAAA TAATACTACA TACAAGTTAT ACTTTCTAGT

1021 CAAAATATAA CATACATNNA TCTTACTTAA CATACCTCAG TTTTAGAGCT ACGGTATGTA
     GTTTTATATT GTATGTANNT AGAATGAATT GTATGGAGTC AAAATCTCGA TGGCATACAT

1081 GAAGAGTCCA TTTCTTATTA GGTAAGTTCC TTTAGTCCTT TTATTACTGG GCACTCTTAA
     CTTCTCAGGT AAAGATAAAT CCATTCAAGG AAATCAGGAA AATAATGACC CGTGAGAATT

1141 TTACATGTAG CTTGAAATAT GTCCAGTTTG AGCAGTGAAC TGAAAATGTC ATGTGATTAA
     AATGTACATC GAACTTTATA CAGGTCAAAC TCGTCACTTG ACTTTTACAG TACACTAATT

1201 GTACATATAT AATTTTTTTT CATAGTAGGT CAATAACCTC CTTTTATTGA CTAATGAATC
     CATGTATATA TTAAAAAAA GTATCATCCA GTTATTGGAG GAAAATAACT GATTACTTAG

1261 AGTTCTCTAA TGATTATACG
     TCAAGAGATT ACTAATATGC
```

FIG. 75A

```
          10          20          30          40          50          60
          |           |           |           |           |           |
  1 AATCAAAATA AAACAGTTAA AGTTTGATTA CTATAATCAA ACACAAAAAA AATGAATATT
    TTAGTTTTAT TTTGTCAATT TCAAACTAAT GATATTAGTT TGTGTTTTTT TTACTTATAA

 61 ATCTTTTATG TCAGTAGAGG GTGAATGAAT CCTTCAGGAT TTTGATGATA GTATCAGATA
    TAGAAAATAC AGTCATCTCC CACTTACTTA GGAAGTCCTA AAACTACTAT CATAGTCTAT

121 CCCAGCACTA TGCTAGAAGT TGTGAAGAAT TCACGAGATG AATAAAATCAC AGATTCTGTC
    GGGTCGTGAT ACGATCTTCA ACACTTCTTA AGTGCTCTAC TTATTTAGTG TCTAAGACAG

181 CTCAAAATGG TTAGATCTAT TCAGGAAACA AAGCTAAAAA AACCCCACCA ATAACTAAAA
    GAGTTTACC AATCTAGATA AGTCCTTTGT TTCGATTTTT TTGGGGTGGT TATTGATTTT

241 ATCAACCAAA TGAAAAACAA CAATCATAAA ATAAGTAAGT ACCTATAGAA AGAAAAGCTC
    TAGTTGGTTT ACTTTTTGTT GTTAGTATTT TATTCATTCA TGGATATCTT TCTTTTCGAG

301 AGAGGAGGTA AAAGATAAC TCTTCCAAAA GGAATACTAT ATACTGTAAA CTGTGTACTG
    TCTCCTCCAT TTTCTATTG AGAAGGTTTT CCTTATGATA TATGACATTT GACACATGAC

361 ATAGAAGGAA GAATTAGAAA NNNNNNNNTG TAAGTGGCAT ACATACTAAG CTAGTGTGAA
    TATCTTCCTT CTTAATCTTT NNNNNNNNAC ATTCACCGTA TGTATGATTC GATCACACTT
```

FIG. 75B

```
421  CACAAGCCTA AATATGTAGT TGCTTCACAG AAGGTTAGAA GTAAATTAAC CTCATGAATT
     GTGTTCGGAT TTATACATCA ACGAAGTGTC TTCCAATCTT CATTTAATTG GAGTACTTAA

481  TCTTGAGAGA ACTTGTAAGG ACTAAGCTTT CGATTTTGGA GAAAGATTTT AATACCAAAT
     AGAACTCTCT TGAACATTCC TGATTCGAAA GCTAAAACCT CTTTCTAAAA TTATGGTTTA

541  AAAAAGTACC TTTGTTTGGT AATCTCAATC ATTATATAATAG TGCTTAGATA ATACCTAGGA
     TTTTTCATGG AAACAAACCA TTAGAGTTAG TAATATTATC ACGAATCTAT TATGGATCCT

601  ACAAATTAAA TATTAAATTT ACTTAAAAAA AAGTACATG ATTGGGGAAT CACAACTGGC
     TGTTTAATTT ATAATTTAAA TGAAATTTTT TTCATGTAC TAACCCCTTA GTGTTGACCG

661  CTTACTAGAT TCTCTNNNNN NATATGCACT GAAAAGAATG AAAAACACTG AACCAAATAT
     GAATGATCTA AGAGANNNNN NTATACGTGA CTTTTCTTAC TTTTGTGAC TTGGTTTATA

721  NTGTTTTTTT AAGTTTAAAA TTAAATTGGA AAAAAATAGT AAGGAATATC AGAAGCAAAA
     NACAAAAAAA TTCAAATTTT AATTTAACCT TTTTTATCA TCCTTATAG TCTTCGTTTT
```

FIG. 75C

```
781  AAATAAAATG AAAGCAAGAA TCCTCAGAGG TAGCACGAAA TTTGGCTTTG CTTAGATGGA
     TTTATTTTAC TTTCGTTCTT AGGAGTCTCC ATCGTGCTTT AAACCGAAAC GAATCTACCT

841  TCTATCAAAG CTATGGCCCA TGAAAAGGAT TCAGGAGTTA GTTTAAAGCT GGTTCACATA
     AGATAGTTTC GATACCGGGT ACTTTCCTA  AGTCCTCAAT CAAATTTCGA CCAAGTGTAT

901  ATGGAATCTA GCAGAAGACT GTGCATAAAG GTGGTCTAAG AACAACAATA TCCTGACCAG
     TACCTTAGAT CGTCTTCTGA CACGTATTTC CACCAGATTC TTGTTGTTAT AGGACTGGTC

961  GTGAGGGGGC TCACNCTNAA TNCCAGCACT TTGGGAGCCC AAGGTGGGTG GATCACGAGG
     CACTCCCCCG AGTGNGANTT ANGGTCGTGA AACCCTCGGG TTCCACCCAC CTAGTGCTCC

1021 TCAGGAGTTT GAGACCAGCC TGACCAACAT GGTGAAACCG CGTCTCTACT AAAAATAGAA
     AGTCCTCAAA CTCTGGTCGG ACTGGTTGTA CCACTTTGGC GCAGAGATGA TTTTTATCTT

1081 AAATTAGCCG NGCCTACGTG CTTCTAATCC CAGCTGAACT CAGGAGACTG AGACAGGAGA
     TTTAATCGGC NCGGATGCAC GAAGATTAGG GTCGACTTGA GTCCTCTGAC TCTGTCCTCT

1141 ATCACTTGAA CCCAGCATGC AAGCTTNNNN NNGCCACTGC ACTCCAGCCT AGGGTGCAAA
     TAGTGAACTT GGGTCGTACG TTCGAANNNN NNCGGGTGACG TGAGGTCGGA TCCCACGTTT

1201 AAAAAAAAA  ANGACACATT ACTCAGGTAA GGTAATCAAT AA
     TTTTTTTTT  TNCTGTGTAA TGAGTCCATT CCATTAGTTA TT
```

FIG. 76A

```
- AAGGTAAAAATTATCTCTTTTTTTCTCTCCCCCAATGTAAAAAGTTATAG -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- AAGGTAAAAATTATCTCTTTTTTTCTCTCCCCCAATGTAAAAAGTTATAG -

- TGGGTTTTACATGTGTAGAATCATTTTCTTAAAACTTTATGAATACCATT -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- TGGGTTTTACATGTGTAGAATCATTTTCTTAAAACTTTATGAATACCATT -

- ATTTTCTTGTATTCTGTGACATGCCCACCTTACAGAGAGGACACATTTAC -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTTTCTTGTATTCTGTGACATGCCCACCTTACAGAGAGGACACATTTAC -

- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGTAG -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGTAG -

- ATGTTTAGAGTGAACAGAACAAATTTTTCTGTGCTTACAGGTTATGGCTG -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- ATGTTTAGAGTGAACAGAACAAATTTTTCTGTGCTTACAGGTTATGGCTG -

- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -

- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -

- ATTGTTATAAATAATGAGGGAAAACAATTTACACATAGCAAATTTAAAAA -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTGTTATAAATAATGAGGGAAAACAATTTACACATAGCAAATTTAAAAA -

- TTACTGTCATTTGATTTGTTAATATATTTTTCTCTTTAGTGGGAAATTAA -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- TTACTGTCATTTGATTTGTTAATATATTTTTCTCTTTAGTGGGAAATTAA -

- ATTTTAAAAAATTCCCTTTCGACTGTAGAACAAATAGGAATTTGGCCTGT -
```

FIG. 76B

```
|||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTTTAAAAAATTCCCTTTCGACTGTAGAACAAATAGGAATTTGGCCTGT -

- GGGGTCTACTTGCTTATTATATTTGTAAGCTAGTGGTAGGAAATAGCAAA -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- GGGGTCTACTTGCTTATTATATTTGTAAGCTAGTGGTAGGAAATAGCAAA -

- TGCTCACTACCACTAATAAGAACATTTCTAAATCTGATGTTCTGAGGATT -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- TGCTCACTACCACTAATAAGAACATTTCTAAATCTGATGTTCTGAGGATT -

- TTTAGAGCTTATAGTAGCAAAAAGAAAAGGGAAATTCTATCCGAGATGTC -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- TTTAGAGCTTATAGTAGCAAAAAGAAAAGGGAAATTCTATCCGAGATGTC -

- CTTTGTTGTAGGCCTAATGAGAAAAGGTTGAAGATAAAGTTCTGGTACTC -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- CTTTGTTGTAGGCCTAATGAGAAAAGGTTGAAGATAAAGTTCTGGTACTC -

- ATTTAAGTGTAATATTGAAAATTGATATTACCGAATCTGGAACAACCAAT -
  |||||||||||||||||||||||||||||||||||||||||||||||||||
- ATTTAAGTGTAATATTGAAAATTGATATTACCGAATCTGGAACAACCAAT -

- TTAAAATAAGGAAAGAAAGACACTGTGTTTTCT -
  |||||||||||||||||||||||||||||||||
- TTAAAATAAGGAAAGAAAGACACTGTGTTTTCT -
```

FIG. 77A

```
        10         20         30         40         50         60
  1 AGAAAACACA GTGTCTTTCT TTCCTTATTT TAAATTGGTT GTTCCAGATT CGGTAATATC
    TCTTTTGTGT CACAGAAAGA AAGGAATAAA ATTTAACCAA CAAGGTCTAA GCCATTATAG

 61 AATTTTCAAT ATTACACTTA AATGAGTACC AGAACTTTAT CTTCAACCTT TTCTCATTAG
    TTAAAGTTA  TAATGTGAAT TTACTCATGG TCTTGAAATA GAAGTTGGAA AAGAGTAATC

121 GCCTACAACA AAGGACATCT CGGATAGAAT TTCCCTTTTC TTTTTGCTAC TATAAGCTCT
    CGGATGTTGT TTCCTGTAGA GCCTATCTTA AAGGGAAAAG AAAAACGATG ATATTCGAGA

181 AAAAATCCTC AGAACATCAG ATTTAGAAAT GTTCTTATTA GTGGTAGTGA GCATTTGCTA
    TTTTTAGGAG TCTTGTAGTC TAAATCTTTA CAAGAATAAT CACCATCACT CGTAAACGAT

241 TTTCCTACCA CTAGCTTACA AATATAATAA GCAAGTAGAC CCCACAGGCC AAATTCCTAT
    AAAGGATGGT GATCGAATGT TTATATTATT CGTTCATCTG GGGTGTCCGG TTTAAGGATA

301 TTGTTCTACA GTCGAAAGGG AATTTTTTAA AATTTAATTT CCCACTAAAG AGAAAAATAT
    AACAAGATGT CAGCTTTCCC TTAAAAAATT TTAAATTAAA GGGTGATTTC TCTTTTTATA

361 ATTAACAAAT CAAATGACAG TAATTTTTAA ATTTGCTATG TGTAAATTGT TTTCCCTCAT
    TAATTGTTTA GTTTACTGTC ATTAAAAATT TAAACGATAC ACATTTAACA AAAGGGAGTA

421 TATTTATAAC AATTCATACT ACAATTTAAT TTAGTAAACA TTTTGTAGA AAATATTTAA
    ATAAATATTG TTAAGTATGA TGTTAAATTA AATCATTTGT AAAACATCT  TTTATAAATT
```

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FIG. 77B

```
481  AACAAGATA  CTGAAAGTTA  ATATNAAACC  CAGTGCATGC  TTCTTGTAGG  CCACAGCCAT
     TTGTTTCTAT  GACTTTCAAT  TATANTTTGG  GTCACGTACG  AAGAACATCC  GGTGTCGGTA

541  AACCTGTAAG  CACAGAAAA  TTTGTTCTGT  TACTCTAAAC  ATCTACACTG  GCCAAATTCC
     TTGGACATTC  GTGTCTTTT  AAACAAGACA  ATGAGATTTG  TAGATGTGAC  CGGTTTAAGG

601  AATGCTCGAA  TTTAACCCCG  GGATATAACC  TAGTAAATGT  GTCCTCTCTG  TAAGGTGGGC
     TTACGAGCTT  AAATTGGGGC  CCTATATTGG  ATCATTTACA  CAGGAGAGAC  ATTCCACCCG

661  ATGTCACAGA  ATACAAGAAA  ATAATGGTAT  TCATAAAGTT  TTAAGAAAAT  GATTCTACAC
     TACAGTGTCT  TATGTTCTTT  TATTACCATA  AGTATTTCAA  AATTCTTTTA  CTAAGATGTG

721  ATGTAAAACC  CACTATAACT  TTTTACATTG  GGGGAGAGAA  AAAAAGAGAT  AATTTTTACC
     TACATTTTGG  GTGATATTGA  AAAATGTAAC  CCCCTCTCTT  TTTTTCTCTA  TTAAAAATGG

781  TT
     AA
```

FIG. 78A

```
                 10         20         30         40         50         60
                 |          |          |          |          |          |
  1  GATGCTATTT GGGCAATTTC TTATTGACAG TTTTGAAATG TTAGGCTTTT ATCTCCATTT
     CTACGATAAA CCCGTTAAAG AATAACTGTC AAACTTTAC AATCCGAAAA TAGAGGTAAA

 61  TTTAGTACTT AAATTTCCA ACATGGGTGT TCCTTGTTAT TTTATCAGTA TAAAATAGAA
     AAATCATGAA TTTAAAGGT TGTACCCACA ACGAACAATA AAATAGTCAT ATTTTATCTT

121  GAGTGGTTCT GTTCTCGAAT TTAGTATATA CATGAGTATC TAGTGTATGT CAGCCATGAA
     CTCACCAAGA CAAGACCTTA AATCATATAT GTACTCATAG ATCACATACA GTCGGTACTT

181  AATGAACCTT TCAGATGTTT AACTTCAGGG AACCTAAATG AGTCATTGCT CCAGACATTG
     TTACTTGGAA AGTCTACAAA TTGAAGTCCC TTGGATTAAC TCAGTAACGA GGTCTGTAAC

241  TTGCTTTGAA CCCACTATAT TNNNNNNCT CGGGCAATGA CTCAGTGTGG CAAGGATACT
     AACGAAACTT GGGTGATATA ANNNNNNNGA GCCCGTTACT GAGTCACACC GTTCCTATGA

301  ACTGCAGGCC TGTTTCTGGA AGGCACTGGA CTCCCTCTGAT GCAAACTTTG GCCAGGGACT
     TGACGTCCGG ACAAAGACCT TCCGTGACCT GAGGAGACTA CGTTTGAAAC CGGTCCCTGA

361  CCTTGATAGC TCTTAAATAG ATGCTGCACC AACACTCTCT TTCTTTTCTT TCTTTTTCTT
     GGAACTATCG AGAATTTATC TACGACGTGG TTGTGAGAGA AGAAAAGAG AGAAAAGAA
```

FIG. 78B

```
421  TATTCAATAT TAGACTACAA GCAGTCTAAG GACTTCTCAG GGTTTCTAGC TCTCTCTCAT
     ATAAGTTATA ATCTGATGTT CGTCAGATTC CTGAAGAGTC CCAAAGATCG AGAGAGAGTA

481  TTCACACATG CTTTCCTAGT AATCTCTACT CAIATATCTT ACTGCTACGC TGGGGCCAGA
     AAGTGTGTAC GAAAGGATCA TTAGAGATGA GTATATAGAA TGACGATGCG ACCCCGGTCT

541  TAACNNNNNN CTTCCATTTT GTTTTTATCT CTATTCTTCT TCCCCTTCTG CTTTCATTAT
     ATTGNNNNNN GAAGGTAAAA CAAAAATAGA GATAAGAAGA AGGGGAAGAC GAAAGTAATA

601  TGAAACTTTC TGCTTTCATT ATTGAAACTT TCCCAGATTT GTTCTGCTTA ACCTGGCATT
     ACTTTGAAAG ACGAAAGTAA TAACTTTGAA AGGGTCTAAA CAAGACGAAT TGGACCGTAA

661  GGAACTGTTT CCTCTTCCCT GTGCTGCTTT CTCCCATTGC CATGTCCTTT TTTTTTTTTT
     CCTTGACAAA GGAGAAGGGA CACGACGAAA GAGGGTAACG GTACAGGAAA AAAAAAAAAA

721  TTTTTTTTTT TGAGACAGTG TCACTCTGTT GCCCAGGCTG GAGTGCAATG GTGCAATCTT
     AAAAAAAAAA ACTCTGTCAC AGTGAGACAA CGGGTCCGAC CTCACGTTAC CACGTTAGAA
```

FIG. 78C

```
 781  GGCCACTGCA  ACCCCCGCCT  CCCGGGTTCA  AGTGATTCTC  CTGCCTCAGC  CTCCTGAGTA
      CCGGTGACGT  TGGGGGCGGA  GGGCCCAAGT  TCACTAAGAG  GACGGAGTCG  GAGGACTCAT

 841  GCTGGGATTA  CAGGTGCCCA  CCACTATGCC  CGGCTGATTT  TTGTATTTTT  AGTAGAGATN
      CGACCCTAAT  GTCCACGGGT  GGTGATACGG  GCCGACTAAA  AACATAAAAA  TCATCTCTAN

 901  NNNNNNNTTT  CACCATNGCT  GATCAGGCTG  GTCTCGAACT  CCTGACCGCA  GTGANTCCGC
      NNNNNNNAAA  GTGGTANCGA  CTAGTCCGAC  CAGAGCTTGA  GGACTGGCGT  CACTNAGGCG

 961  CCTCCTTGGC  CTCCCAAAGT  GCTGACATTA  CAGGCATGAG  TCACTGCGNC  CAGCCACCAT
      GGAGGAACCG  GAGGGTTTCA  CGACTCTAAT  GTCCGTACTC  AGTGACGCNG  GTCGGTGGTA

1021  TATTCTCTAG  AGGTGAGAGA  ACACTGGCTC  TTCTAACAAG  TTGAAATTTG  ATAGAGACC
      ATAAGAGATC  TCCACTCTCT  TGTGACCGAG  AAGATTGTTC  AACTTTAAAC  TATCTCTGG
```

FIG. 79A

```
            10         20         30         40         50         60
 1  CACAAAAAA GATTATTAGC CACAAAAAAA CCTTGAAGTA AGGCATTAA ATGTTAATGG
    GTGTTTTTT CTAATAATCG GTGTTTTTT GGAACTTCAT TGCGTAATTT TACAATTACC

61  ATTCACTTTA TTGAGCATCT GCTCATATA CTTTAATGAG TGCAAAGTGC TTTGAATATA
    TAAGTGAAAT AACTCGTAGA CGAGTATTAT GAAATTACTC ACGTTTCACG AAACTTATAT

121 ATACGTCATT CAAACCTTAC CATAATCTG AGGAATTGCT ACCTCCACTT CACAGATGGG
    TATGCAGTAA ATTGGAATG GTATTAAGAC TCCTTAACGA TGGAGGTGAA GTGTCTACCC

181 GCACAGGAGG CTTAGATAAC ATGCCCAAAG TCATGCTTCT AGTAAATGGA TATAATTAAG
    CGTGTCCTCC GAATCTATTG TACGGGTTTC AGTACGAAGA TCATTACCT ATATTAATTC

241 ATTCAAATTA TTGATAAGAA TTTGATCTGC CTTACCAGTA TCTAGTAGTA AATCTAAAAG
    TAAGTTTAAT AACTATTCTT AAACTAGACG GAATGGTCAT AGATCATCAT TTAGATTTTC

301 CGCTTTCCAG AGCATGTGCT GTTGATAGAG CTTGATGTGCT AACTCTCTGA AATTTTCCAT
    GCGAAAGGTC TCGTACACGA CAACTACTC GAACTACAGA TTGAGAGACT TTAAAAGGTA

361 TCTTATTTGT CTCACTGGTA TATAGTTATT TTTACTACT TTCATACACC TACTAAGAAG
    AGAATAAACA GAGTGACCAT ATATCAATAA AAATGATGA AAGTATGTGG ATGATTCTTC
```

FIG. 79B

```
421  ACAGGAGGAT  CAAAGATAGG  ATTTCATTTA  GAATGCCTAA  AGCTTCACGT  ATTTAATTC
     TGTCCTCCTA  GTTTCTATCC  TAAAGTAAAT  CTTACGGATT  TCGAAGTGCA  TAAAATTAAG

481  AGAATAAGAT  TCAGGCAGAC  CACCAGTATA  TGCCATGGTC  CCTGGTTATC  TTTCAGCAGG
     TCTTATTCTA  AGTCCGTCTG  GTGGTCATAT  ACGGTACCAG  GGACCAATAG  AAAGTCGTCC

541  TGACCGAGAA  AGAAAACATG  GTAATGTTTA  TGAAATGGTG  GGTTCTTCTA  GTTTCACTTC
     ACTGGCTCTT  TCTTTTGTAC  CATTACAAAT  ACTTACCAC   CCAAGAACAT  CAAAGTGAAG

601  AACATATCTG  CCTTTACTGT  ATTAAGATGA  TGGATTAACT  TATTCTTGAT  ATGGGCATGT
     TTGTATAGAC  GGAAATGACA  TAATTCTACT  ACCTAATTGA  ATAAGAACTA  TACCCGTACA

661  AAAACAATAT  ACTTTTACTA  AACAGCTACA  GAGAGACAAA  TGTGTTTCCA  GACAAACTTA
     TTTTGTTATA  TGAAAATGAT  TTGTCGATGT  CTCTCTGTTT  ACACAAAGGT  CTGTTTGAAT

721  AGAGACTGAG  TGTTCAAACT  GAATAATCTC  GACCTTAATT  GTAACTATAT  TTTATGAAAT
     TCTCTGACTC  ACAAGTTTGA  CTTATTAGAG  CTGGAATTAA  CATTGATATA  AAATACTTTA
```

FIG. 79C

```
 781  CCAGCTGTAA GGCAAAAACA GACTTCTTTG GGCCTACCAC GGGCATTTTG TTCCTGTTAN
      GGTCGACATT CCGTTTTTGT CTGAAGAAAC CCGGATGGTG CCCGTAAAAC AAGGACAATN

 841  NNNTACTCCA AACCTTAAAC CCACGTCCAC TTAAATAATG GCCTGGAAAT AAATGTCATT
      NNNATGAGGT TTGGAATTTG GGTGCAGGTG AATTTATTAC CGGACCTTTA TTTACAGTAA

 901  ATCTGATATT ATACTGAGAT GTTTAGTTAT GAAATCAAAA GTGGAGAATT TCAATCTGTC
      TAGACTATAA TATGACTCTA CAAATCAATA CTTTAGTTTT CACCTCTTAA AGTTAGACAG

 961  CTGTAAGCTT TCTCTGCGGT CACGACCCTC ATGCACTCAG GCTGTGCGGT GCAGCATGCT
      GACATTCGAA AGAGACGCCA GTGCTGGGAG TACGTGAGTC CGACACGCCA CGTCGTACGA

1021  CTGTCATGTC TGTTTTCTTC TGCCTGTACA CGGGTGGTTG TTCCTGTCTA CCTGTTTGAG
      GACAGTACAG ACAAAAGAAG ACGGACATGT GCCCACCAAC AAGGACAGAT GGACAAACTC

1081  GAAATATGAA TACGTNNNNN NCTAGAATCT ACTGCACATG CAATAAGGAA ACAATCAGTA
      CTTTATACTT ATGCANNNNN NGATCTTAGA TGACGTGTAC GTTATTCCTT TGTTAGTCAT

1141  AGAATCACTT TCTCGTGGAA AATTCATTAG AATTAACATC TCGTTTTAAA ATGCTCTATC
      TCTTAGTGAA AGAGCACCTT TTAAGTAATC TTAATTGTAG AGCAAAATTT TACGAGATAG
```

FIG. 79D

```
1201 AAAGTGTAAA TAATTCCTCT CTCTTTCCC TTTTTCACTA AGGAGTTTGT ATATTAAACA
     TTTCACATTT ATTAAGGAGA GAGAAAAGGG AAAAAGTGAT TCCTCAAACA TATAATTTGT

1261 GAATTTCAAG TAATGTATTA TAAATTTATT TAANNTATTT ACAATAAAAT GCCACGTATA
     CTTAAAGTTC ATTACATAAT ATTAAATAA ATTNNATAAA TGTTATTTTA CGGTGCATAT

1321 AGCATCAAGC AACATGANNN NNNCATTGGT AGAAAGCACA ATACATAGTC AAAACAGCAG
     TCGTAGTTCG TTGTACTNNN NNNGTAACCA TCTTTCGTGT TATGTATCAG TTTTGTCGTC

1381 AGTATTAAAT AAACAGAAAA TTTGCAAAAG GCAAGTAAAG AATATACATA TACTTAATTA
     TCATAATTA TTTGTCTTTT AAACGTTTTC CGTTCATTTC TTATATGTAT ATGAATTAAT

1441 TACATAAAAT ATTGATACAG GAGGTAGAAA GAAATTTAGT AAGCAGATAA TGGGGGCAAC
     ATGTATTTTA TAACTATGTC CTCCATCTTT CTTTAAATCA TTCGTCTATT ACCCCCGTTG

1501 AGAGTCCTCA GCAGAGCTTC CCTTCTAACA AAAAGCAGCC CAATAAATTA TTTTTTTTT
     TCTCAGGAGT CGTCTCGAAG GGAAGATTGT TTTTCGTCGG GTTATTTAAT AAAAAAAAA

1561 CTAACAAAAA GCAGCCTGAA AAATCGAGCT GCAAACATAG ATTAGCAATC GGCTGAAAGT
```

FIG. 79E

```
      GATTGTTTT CGTCGGACTT TTTAGCTCGA CGTTTGTATC TAATCGTTAG CCGACTTTCA

1621  GCGGGAGAAT GCTGGCAGCT GTGCCAATAG TAAAGGGCTA CCTGGAGCCG GGCGCGTGGC
      CGCCCTCTTA CGACCGTCGA CACGGTTATC ATTTCCCGAT GGACCTCGGC CCGCGCACCG

1681  TCACGCTGTA ATCCCAGCAC TTTGGGAGGG CGAGGCAACG CGGATCACCT GAGGTCGGGA
      AGTGCGACAT TAGGGTCGTG AAACCCTCCC GCTCCGTTGC GCCTAGTGGA CTCCAGCCCT

1741  GTTTGAGATC AGCCCGACCA ACATGGAGAA ACCCCGTCTC TACTAAAAAA AAAAAAAAAA
      CAAACTCTAG TCGGGCTGGT TGTACCTCTT TGGGGCAGAG ATGATTTTTT TTTTTTTTTT

1801  AAAGGCAAAA AATGAGCCGG GCATGGTGGC ACATGCCTTG CACATCCCAG CTGAGGCAGG
      TTTCCGTTTT TTACTCGGCC CGTACCACCG TGTACGGAAC GTGTAGGGTC GACTCCGTCC

1861  AGAATTCACT TGAACCTGGG AGGTAGAGAT TGCGGTGAAG CGAGATCACG TCATTGCACT
      TCTTAAGTGA ACTTGGACCC TCCATCTCTA ACGCCACTTC GCTCTAGTGC AGTAACGTGA

1921  CCAGCCTGGG CAAAAGAGC AAAACTTAGT CTCAAAAAAA AAAANNCAAA GAAAAAA
      GGTCGGACCC GTTTTTCTCG TTTGAATCA GAGTTTTTT TTTNNGTTT CTTTTTT
```
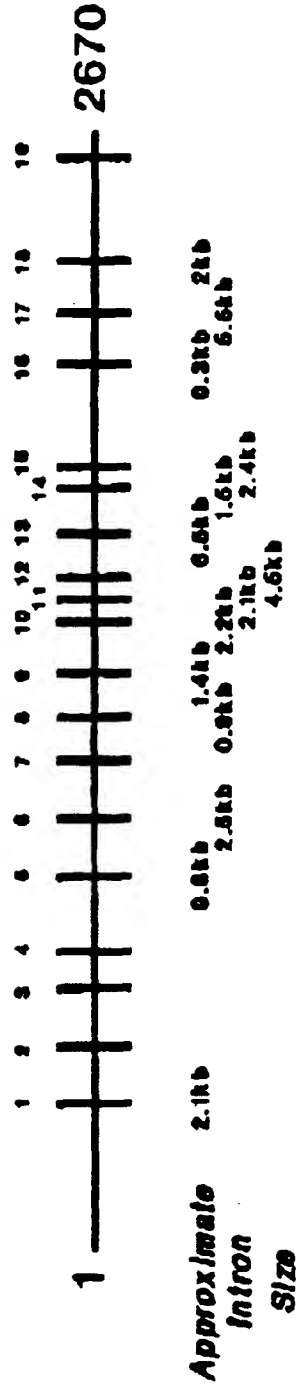
FIG. 80

Genomic Organization of PSM Gene

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C12N 15/12, 15/64; C12Q 1/68; C07K 14/435
US CL   :536/23.5; 435/6, 7.1, 320.1, 252.3, 69.3; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/6, 7.1, 320.1, 252.3, 69.3; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

INPADOC, CA
search terms: prostate specific membrane antigen

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 94/09820 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 11 May 1994, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier document published on or after the international filing date
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"O"	document referring to an oral disclosure, use, exhibition or other means
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"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 APRIL 1996	14 MAY 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	ANTHONY C. CAPUTA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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